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# PREFACE

For several years the Editorial Committee has been considering the advisability of including the titles of articles cited in the bibliographies of the *Annual Review of Entomology*. The Committee has long felt that this would be a valuable addition if ways could be found to accomplish it economically and without substantial loss of text. Although it developed that neither of these objectives could be wholly met, the Committee, at its 1959 meeting, voted to use bibliographic titles beginning with Volume 7 (1962). In the meantime, the Entomological Society of America and a number of entomologists in countries other than the United States and Canada have forwarded requests and suggestions concerning the inclusion versus the omission of titles in the lists of literature cited. The problem is a complex one and not as easy to solve as many may think. Accordingly, we feel that a fuller explanation at this time might aid in gaining a general understanding of the matter and assure readers of the *Review* that steps have been taken to solve the problem.

With the inclusion of titles, a typical page of bibliography would contain 21.5 entries, instead of 38.4 as in the past. A typical volume of the *Annual Review of Entomology* contains approximately 2750 references, which have appeared in 71.5 printed pages. Since the inclusion of titles would permit only 21.5 citations per page, the space devoted to bibliographies would be increased to 121 pages. For instance, in Volume 4, which is a typical volume, there would have been 313 pages of text plus 121 of bibliography, instead of 363 pages of text and 71 of bibliography. The question before the Committee, therefore, was whether or not entomologists, and others who read the *Review*, would want a publication in which 30 per cent of the effective space is used for references rather than for text. Some readers have told us that they feel it would be wasteful to use so much space for the titles of articles which, in any case, can be found from the information given in the reference citations as they are now presented. Should the *Review* endorse and participate in this bibliographic service? Inasmuch as we are bound by page limitations set by the publisher, would our readers care to have three of the articles sacrificed to permit the inclusion of titles in those that remain?

Other matters also had to be borne in mind: In the *Review* we have bibliographies of considerable length, whereas in scientific journals the number of citations is ordinarily relatively few. Then, there is the matter of increased time and money associated with the checking and editing of references that include titles. This is in addition to mounting costs in recent years for printing and manufacturing. Subscriptions to the *Annual Review of Entomology* are barely enough to cover the costs of printing and binding. Some of our correspondents have suggested that to compensate for the additional space required for titles, the price of the volume be increased by one dollar. It is doubtful that this would cover the costs of the required



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additional pages and printing. Moreover, Annual Reviews, Inc., is reluctant to increase the sales price of the *Review* until all other possibilities of effecting reasonable economies have been exhausted. It has also been suggested that only abbreviated titles be used. However, the Editorial Committee felt that this would be an unsatisfactory solution for a number of reasons, among which would be the tremendously increased editorial work involved. (Few readers are aware of the extent of revision and editing of bibliographies required for most manuscripts.)

After considering all aspects of the situation, after consulting with entomologists with different interests, and after obtaining the approval of the Governing Board of Annual Reviews, Inc., the Editorial Committee, as we have already indicated, decided to permit the inclusion of titles in the bibliographies even though this will reduce the number of pages of text in each volume. In addition, beginning with the present volume, a new double-column reduced type-size format is being used for the bibliographies. The space saved by this change will, in part, help provide space for titles. Of course, were titles not to be used, the same saving of space could be used for more text. In any event, it is hoped that the inclusion of titles and the change in format are in accord with the wishes of most of the readers of the *Review* and that it will be an added service to entomologists seeking comprehensive reviews of subjects of their interest. We shall appreciate hearing from any of our readers concerning the matter after enough volumes have appeared with titles to enable a comparison to be made.

Again, the Editors and the Editorial Committee wish to express their deep thanks and sincere appreciation to our Editorial Assistant, Mrs. Betty Schink, who has performed exceptionally yeomanly service in handling many of the editorial details involved in the production of the present volume.

R.G. L.E.R.
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# DARWIN'S CONTRIBUTIONS TO ENTOMOLOGY

By Jeanne E. Remington and Charles L. Remington
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I feel like an old war-horse at the sound of the trumpet when I read about the capturing of rare beetles—is not this a magnanimous simile for a decayed entomologist? It really almost makes me long to begin collecting again. . . . "Floreat Entomologia"! —to which toast at Cambridge I have drunk many a glass of wine. So again, "Floreat Entomologia." N.B. I have *not* now been drinking any glasses full of wine. Yours, C.D.

The foregoing quotation is from a letter (18) to Sir John Lubbock by an Original and Life Member of the Entomological Society of London in the middle years of his life. Charles Darwin thus thought of himself as at least partly an entomologist, and his knowledge of insects and keen interest in them led to many of his conclusions on evolution and natural selection.

Darwin's entomological interests began in boyhood, as described by him in his autobiographical notes (18):

I must have observed insects with some little care, for when ten years old (1819) I went for three weeks to Plas Edwards on the sea coast in Wales, I was very much interested and surprised at seeing a large black and scarlet Hemipterous insect, many moths (Zygaena), and a Cicindela which are not found in Shropshire. I almost made up my mind to begin collecting all the insects which I could find dead, for on consulting my sister I concluded that it was not right to kill insects for the sake of making a collection.

When he went to the University of Cambridge from 1828 to 1831, Darwin spent much of his non-academic time in collecting beetles, visiting entomologists, and going on collecting trips. One of the visits was to the Reverend F. W. Hope, who later established the professorship of entomology at the University of Oxford. Darwin wrote that on that visit they "did little else but talk about and look at insects." Hope complimented Darwin's "exertions in Entomology," and said that he and his friends had "taken a wonderfully great number of good insects . . . ." In July, 1829, the two made a week's tour through North Wales to collect insects.

Among Darwin's closest college friends were W. Darwin Fox, his second cousin, and Leonard Jenyns, both of whom were entomologically inclined. In 1831, Jenyns wrote to J. F. Stephens, a prominent British entomologist:

. . . Darwin, I am sorry to say, has taken his degree, so that we are likely to lose him soon at Cambridge altogether, which I shall very much regret, he having assisted me greatly in working out the Cambridgeshire insects, and proved so very diligent a collector; though I have not yet been able to induce him to extend his inquiries beyond the order of Coleoptera. He comes up for one more term this spring, I believe solely for entomological purposes . . . [18].

That final spring did much to broaden Darwin's entomological background,

chiefly through his very close and continuous contact with Professor Henslow, as noted below.

Darwin's letters and autobiography of this period are full of references to collecting, e.g.,

> But no pursuit at Cambridge was followed with nearly so much eagerness or gave me so much pleasure as collecting beetles. It was the mere passion for collecting, for I did not dissect them, and rarely compared their external characters with published descriptions, but got them named anyhow. I will give a proof of my zeal: one day, on tearing off some old bark, I saw two rare beetles, and seized one in each hand; then I saw a third and new kind, which I could not bear to lose, so that I popped the one which I held in my right hand into my mouth. Alas! it ejected some intensely acrid fluid, which burnt my tongue so that I was forced to spit the beetle out, which was lost, as was the third one. . . . No poet ever felt more delighted at seeing his first poem published than I did at seeing, in Stephens' "Illustrations of British Insects," the magic words, "captured by C. Darwin, Esq." [18].

In Cambridge at that time, students interested in any field of science were accustomed to meet once a week to talk and exchange information and specimens at the home of the professor of botany, John Stevens Henslow. Fox and Jenyns, Darwin's "brother entomologists," took him to these meetings, where Darwin and Henslow became acquainted and eventually became very close friends. This friendship, Darwin wrote in his autobiography, "influenced my whole career more than any other." One of the influences was the broadening of Darwin's entomological interests and knowledge. Henslow himself had become interested in insects at the same youthful age that Darwin had and collected and observed insects all his life, contributing records to British insect monographs and also writing some small papers on insects himself. He was especially interested in Hymenoptera and studied the construction of nests and parasitic larvae found in them (e.g., his paper on this subject, in *The Zoologist* for 1849, contains the first British record for the beetle *Velleius dilatatus* Fabricius). Another paper of his was on the feeding habits of some microlepidoptera, and still another, on the life history of the wheat midge.

Henslow was an enthusiastic naturalist with a broad knowledge of several fields of natural history. If there had been an opening at the University for teaching entomology, we can speculate that this might have been his first choice, but there was not. Henslow held the professorship of mineralogy from 1822 to 1828 and that of botany for many years, commencing in 1825. Most of his published work was on botany.

Henslow's biographer (19) states that he "did not content himself with mere collecting; he was a close observer also of the habits of animals," and Darwin wrote that Henslow's "strongest taste was to draw conclusions from long continued minute observations." Darwin's own genius was to appear a few years later in the form of brilliant syntheses of long-continued observations, by himself and from the published works of hundreds of other biolo-

gists. Henslow's point of view and his broad knowledge of natural history were of inestimable value to Darwin when he joined Henslow and the group of young Cambridge naturalists on their frequent field trips. Darwin absorbed much information on insects, as well as other animals and plants, and developed his powers of observation and inductive reasoning. His progress is indicated by the fact that in August, 1831, when Henslow was invited to recommend a young naturalist for the voyage of the *Beagle*, he named Darwin and wrote to him (18):

> I have stated that I consider you to be the best qualified person I know of who is likely to underake such a situation. I state this not in the supposition of your being a *finished* naturalist, but as amply qualified for collecting, observing, and noting, anything worthy to be noted in Natural History.

As the sole naturalist with the *Beagle*, Darwin was responsible for all branches of natural history. His interest and knowledge of insects other than beetles thus inevitably grew, as shown by his large collections in British museums and by a score or more references to insects in the *Journal of Researches into the Geology and Natural History of the Various Countries Visited by H. M. S. Beagle* (2). These references cover such diverse aspects as bird parasites being carried by their hosts to other localities, the behavior of Lampyridae, the flipping of elaterid beetles, noise-producing butterflies, army ants, *Pepsis* wasps stinging and storing spiders for larval food, effect of heat on insects in usually cool Montevideo, mass migrations at sea of *Colias* butterflies, insects found in bird stomachs, the difference in the insect fauna of various places, locust swarms, ant lions, and bites of reduviids. On the advantages to a naturalist of such a trip, he wrote:

> Moreover as a number of isolated facts soon become uninteresting, the habit of comparison leads to generalization. On the other hand, as the traveller stays but a short space of time in each place, his descriptions must generally consist of mere sketches, instead of detailed observation. Hence arises, as I have found to my cost, a constant tendency to fill up the wide gaps of knowledge, by inaccurate and superficial hypotheses.

But the feeling of sketchiness stimulated him to further observation, reading, and correspondence on natural history to fill up the gaps and provide a sounder, broader base for his hypotheses.

Although Darwin did not work on insect taxonomy, his eight years (1846 to 1854) of monographing the Cirripedia (barnacles) gave him a background that later enabled him to discuss the principles of a natural classification, based on evolutionary affinity and not adaptive similarity. He thus had an appreciation for the labor of taxonomy; after repeatedly rewriting a manuscript, trying to decide whether a particular form of barnacle was a species or variety, he "gnashed [his] teeth, cursed species, and asked what sin [he] had committed to be so punished." But he afterward felt that it had been valuable training.

In his book *On the Origin of Species* (6), there are about 50 references to insects, varying from a line or two up to 15 solid pages, as illustrations of the struggle for existence, natural selection, or natural classification. These may be divided into three types. In one category, there are simply references to generally known facts, such as the resemblance between the larval stage of flies, beetles, moths, etc., the useless hind wings of some beetles, that certain flies lay a large number of eggs, or that Linnaeus mistook a certain homopteran for a moth. Secondly, there are references to original data derived from entomological writings. Darwin had a thorough acquaintance with the entomological literature of that time and often wrote to the authors when there were questions or suggestions which occurred to him when reading. As examples of this second type, in the *Origin*, there are several references to Wollaston's work on Madeira insects, as when Darwin explained the apterous condition of many beetles as an instance of obvious natural selection. Darwin referred disapprovingly to Wollaston's conclusions in a private letter to Hooker in 1855 (18):

> . . . I have just finished working well at Wollaston's "Insecta Maderensia": it is an *admirable* work. There is a very curious point in the astounding proportion of Coleoptera that are apterous; and I think I have guessed the reason, viz. that powers of flight would be injurious to insects inhabiting a confined locality, and expose them to the sea: to test this, I find that the insects inhabiting the Dezerte Grande, a quite small islet, would be still more exposed to this danger, and here the proportion of apterous insects is even considerably greater than on Madeira proper. Wollaston speaks of Madeira and the other Archipelagoes as being "sure and certain witnesses of Forbes' old continent" [Atlantis], and of course the Entomological world implicitly follows this view. But to my eyes it would be difficult to image facts more opposed to such a view. It is really disgusting and humiliating to see directly opposite conclusions drawn from the same facts.

But in the *Origin* Darwin uses only Wollaston's facts, with abundant acknowledgment, and gives his own conclusions. Darwin also refers to Fabre's work, to Lyell's finding a shell attached to the leg of a *Dytiscus* as an example of distribution methods, to the development of *Aphis* as given by Huxley, to Westwood's observation on the constancy of structure of Hymenoptera antennae, and so forth. Having these various facts brought into the discussion of evolution and selection could only stimulate the entomologists who had made them to further observations to refute or to sustain the generalizations that Darwin had drawn from their data.

The third type of entomological reference in the *Origin* includes those that are based entirely on Darwin's own observations or on his extensive corroborative evidence added to another's data. Examples of his own individual work are his remarks on the similarity of British and Brazilian fresh water insects and on the mutual evolution of flowers and insect pollinators. The confirmatory references concern such material as Pierre Huber's discovery of slave-making habits in a Swiss *Formica* (*Polyerges*) ant and F. Smith's study

of a British species of slave-maker, but Darwin supplemented this by detailed observations of his own: "During the months of June and July, on three successive years, I have watched for many hours several nests in Surrey and Sussex . . . ." He wrote further to Joseph Hooker:

> I have had some fun here in watching a slave-making ant; for I could not help rather doubting the wonderful stories, but I have now seen a defeated marauding party, and I have seen a migration from one nest to another of the slave-makers, carrying their slaves (who are *house*, and not field niggers) in their mouths!

The chapter also gives original comparisons on the habits and behavior of the British and Swiss species. Since the Swiss ants were much more dependent on their slaves than the British species, Darwin conjectured that they were at a more advanced stage in the evolution of the slave-making instinct.

In discussing the cell-making instincts of the hive bee, Darwin starts by saying: "I was led to investigate this subject by Mr. Waterhouse, . . . and the following view may, perhaps, be considered only as a modification of his theory." He had written to Wallace (January, 1859) that bees' combs were a special hobby of his and that he thought he could throw some light on the subject. Darwin's additions were much more than just a modification of Waterhouse's data and theory. Darwin showed the gradation of the nest-making ability from the simple nest of bumble bees, to the intermediate type of a Mexican *Melipona*, to the most intricate architecture of the hive bee nest, which is geometrically ideal for holding the greatest amount of honey with the least consumption of wax. Thus, he was able to explain the possible evolution of this instinct, even though he had once shown discouragement in a letter to Hooker (18):

> I have partly written this note to drive bee's-cells out of my head; for I am half-mad on the subject to try to make out some simple steps from which all the wondrous angles may result.

He acknowledged the experiments of Tegetmeier but went on further with the experiments himself, putting colored wax into bees' hives and closely following their methods. He wrote:

> Some of these statements differ from those made by the justly celebrated elder Huber, but I am convinced of their accuracy; and if I had space, I would show that they are comformable with my theory.

In the same chapter on "Instinct," through the pages of description of sterile females in ant nests, Darwin frequently mentions the observations of F. Smith and supports them by saying: "I have myself compared perfect gradations of this kind," and further, "I can affirm that the eyes are far more rudimentary in the smaller workers than can be accounted for merely by their proportionally lesser size." He also gives his own observations and concludes with hypotheses based on both.

Darwin's third book, which deals extensively with entomology, is *The*

*Descent of Man and Selection in Relation to Sex* (17). This book appeared in 1871, 12 years after the *Origin* and 26 years after the *Journal of Researches*. The increase in entomology in each of the three successive books is correlated with its date and shows that Darwin found that insects exhibited characters and habits of such variety that they were extremely useful in discussions of the laws of natural selection. For example, in the *Descent* there are seven pages on insects in the consideration of sex ratios. His growing acquaintance with the literature and with entomologists personally, mostly through correspondence, is shown in this book by the fact that 85 separate entomologists are named, some many times, and their data are used in the two chapters on secondary sexual characters of insects. These men were in the British Isles, Germany, North and South America, Australia, Africa, and perhaps even elsewhere. Very little of it seems to be original observation on Darwin's part, although he made many of the examinations of beetle specimens himself and occasionally mentions his observations of the insects studied while on the *Beagle* voyage or of common English insects. However, he caused much original investigation by asking questions of other persons, who would either do the experiments or make the observations and then communicate with Darwin. He thus was able to use this new material in his writings, with due acknowledgment to the suppliers of information. For example, he states that the eye spots on wings of Lepidoptera are difficult to explain, "for though appearing to us so ornamental, they are never present in one sex and absent in the other, nor do they ever differ much in the two sexes." And he added a footnote: "Mr. [Henry W.] Bates was so kind as to lay this subject before the Entomological Society, and I have received answers to this effect from several entomologists."

Other lines of communication that he used for suggestions and questions are shown in the following. A letter to J. H. Fabre (18) says:

> Allow me to make a suggestion in relation to your wonderful account of insects finding their way home. I formerly wished to try it with pigeons: namely, to carry the insects in their paper "cornets," about a hundred paces in the opposite direction to that which you ultimately intended to carry them; but before turning round to return, to put the insect in a circular box, with an axle which could be made to revolve very rapidly, first in one direction, and then in another, so as to destroy for a time all sense of direction in the insects.

A letter to A. de Candolle (18) posed many questions:

> Finally, will you permit me to ask you a question: have you yourself, or some one who can be quite trusted, observed . . . that the butterflies on the Alps are tamer than those on the lowlands? Do they belong to the same species? Has this fact been observed with more than one species? Are they brightly coloured kinds? I am especially curious about their alighting on the brightly coloured parts of ladies' dresses, more especially because I have been more than once assured that butterflies like bright colours, for instance, in India the scarlet leaves of Poinsettia.

He wrote to A. R. Wallace for much assistance and comment as in a letter in 1867 (18):

> I am hard at work on sexual selection, and am driven half mad by the number of collateral points which require investigation. . . . Can butterflies be polygamous! *i.e.*, will one male impregnate more than one female?

He also wrote to Fritz Müller for information on sexual selection in invertebrates and asked Müller to examine some lamellicorn beetles to study the difference in sexes in noise-making ability.

While none of Darwin's papers or books was exclusively entomological, a number of those not considered above have an obvious bearing on the insect world. His book *The Various Contrivances by which Orchids are Fertilised by Insects* (12) contains a great amount of data on the nectar-feeding and pollinating behavior of insects. In evaluating this book, the modern orchidologist Oakes Ames wrote (1): "Many interesting discoveries have been made since 1862 . . . but the fundamentals on which they rest are contained in Darwin's classic treatment of the broader aspects of insect pollination." Darwin considered the interdependent evolution between orchids and their insect pollinators to be one of the most marvelous examples of natural selection. In the chapters on various orchid species, data on the number and types of insects visiting the flower are given; e.g., for *Orchis pyramidalis* Linnaeus, he lists 23 Lepidoptera species that he had found with pollinia of this orchid attached to them. He also noted that, in some cases, thrips were the chief agent for carrying pollen; in another, heavy bees were necessary to release the trigger mechanism of the pollinia; in two related species of *Epipactis* orchids, it was found that one was pollinated by Diptera (*Coelopa*, *Sarcophaga*), hive bees, *Crabro*, and three other kinds of Hymenoptera, but never bumble bees, whereas the related species was pollinated, and therefore adapted to, only a species of *Vespa*. Darwin described the part of the anatomy of the insects to which the pollinia are attached for transport to the next orchid. He compared groups of orchid plants in adjacent areas and found that the plants in exposed situations were not visited as frequently by their lepidopterous pollinators as the ones in more protected places.

He also wrote several small papers on this subject, with such titles as "On the Agency of Bees in the Fertilisation of Papilionaceous Flowers" (3, 4), "Do the Tineina or Other Small Moths Suck Flowers, and if so What Flowers?" (5), "Fertilisation of the Fumariaceae" (10), and "The Scarcity of Holly Berries and Bees" (14).

In his book *Insectivorous Plants* (11), the types, sizes, and numbers of insects captured by the plants are studied; e.g., insects as light as *Culex* alighting on the outer rays of the sundew (*Drosera*) are enough to start the tentacles moving inward. On the Venus's-flytrap (*Dionaea*) Darwin noted that, of 14 leaves containing arthropods, four were very small (three ants, one fly) and 10 were large (five elaters, two chrysomelids, one *Curculio*, one

spider, and one *Scolopendra*) and, with additional data, concluded that the smaller forms can escape from the trap through the intermeshing bars as it is closing, whereas the larger ones, and especially poor fliers, cannot escape. This is compared to the fact that the sundew captures mostly good fliers, even such comparatively large ones as the butterfly *Coenonympha pamphilus* (Linnaeus).

Probably Darwin's greatest contemporary contribution to the field of entomology was his encouragement of individuals working in the subject. In some of the examples given above, it was shown that he stimulated further experimentation and observation through questions. But beyond that, he was unstinting in his praise and appreciation for the work of others. He wrote cordial, kindly letters to authors of papers he especially liked. When unusually interesting and informative letters were sent to him, he would send them (with permission) to be published as notes in *Nature* with additional comments of his own: "Habits of Ants" (8) was a letter that Darwin had received from a Mr. Hague; "Recent Researches on Termites and Honey Bees" and others (9, 13, 15) were received as letters from F. Müller in Brazil, as was "The Sexual Colors of Certain Butterflies," which is accompanied by Darwin's comments on courtship (16).

One of the naturalists most strongly praised and encouraged, both in private letters and in print, was Henry W. Bates, the entomologist who first described clearly the phenomenon of mimicry. Bates' paper, entitled "Contributions to an Insect Fauna of the Amazon Valley" was published in 1862, and Darwin wrote to him as follows:

> Dear Bates,—I have just finished, after several reads, your paper. In my opinion it is one of the most remarkable and admirable papers I ever read in my life. The mimetic cases are truly marvellous, and you connect excellently a host of analogous facts. The illustrations are beautiful, and seem very well chosen. . . . I am rejoiced that I passed over the whole subject in the "Origin," for I should have made a precious mess of it. You have most clearly stated and solved a wonderful problem. . . . I have one serious criticism to make, and that is about the title of the paper; I cannot but think that you ought to have called prominent attention in it to the mimetic resemblances. Your paper is too good to be largely appreciated by the mob of naturalists without souls; but, rely on it, that it will have *lasting* value, and I cordially congratulate you on your first great work.

Darwin also praised it highly in a five-page review (7) with such statements as:

> Mr. Bates has given to these facts the requisite touch of genius, and has, we cannot doubt, hit on the final cause of all this mimicry. . . . It is hardly an exaggeration to say, that whilst reading and reflecting on the various facts given in this Memoir, we feel to be as near witnesses, as we can ever hope to be, of the creation of a new species on this earth.

He repeated a great deal of his review almost verbatim in later editions of the

*Origin* and devoted three pages to the subject in the *Descent of Man and Sexual Selection.*

Upon his return from the voyage of the *Beagle*, Darwin often attended the meetings of the Entomological Society of London. In 1838, he was elected a member of the Council and a Vice President and presided over the General Meeting in April, 1838, and a few other meetings. Because of ill health and living away from London, he did not take an active part in the Society in his later years, but he retained his interest in it and remained a lifelong member (20). In 1863, he wrote to Lyell of his trust that the theory of evolution would eventually prevail, "but this result, I begin to see, will take two or three lifetimes. The entomologists are enough to keep the subject back for half a century." In 1867, Darwin wrote to Professor Ernst Haeckel:

> No body of men were at first so much opposed to my views as the members of the London Entomological Society, but now I am assured that, with the exception of two or three old men, all the members concur with me to a certain extent [18].

Nevertheless, E. B. Poulton, writing in 1901 (21), felt that even ten years after these words were written there was little improvement, but, by 1887, twenty years later, there was a tremendous difference, and Darwin's theories were largely accepted by the British entomologists.

It is not clear why the entomologists should have been so opposed, although one can speculate on two possible reasons when looking through the published proceedings of the London society in the 1860's. First was the disputatious atmosphere of the group at that time. J. W. Douglas, in his presidential address at the end of his 1860 term, denounced the "personal altercation and recrimination of entomologists, . . . "Genus irritabile" is an appellation which bids fair, if this spirit be not repressed, to attach with truth to entomologists as a body . . . ." A clue to the cause of the disagreeableness may be found in another part of the same address:

> Collecting has with some persons become such a mania that to form a collection, at whatever cost, appears to be the sole object of their lives. The possessor of a unique specimen prides himself upon having it; the captor of rarities hoards them up, or barters them away with reference to their money value; and others take advantage of the ignorance of young collectors to obtain from them any rare species they may happen to possess. My attention has been so often called to cases of want of honourable feeling and consideration . . . that I feel bound to give expression to the disgust of all right-minded entomologists at such conduct.

A second reason for the opposition may have been that the already irritable collecting-type entomologists did not wish to have their *status quo* disturbed by the Darwinian emphasis on becoming deeper naturalists and observers and on the need for serious study, close attention to details, and inductive analysis. Darwin wrote to Bates in 1861 (18):

> I can understand that your reception at the British Museum would damp you; they are a very good set of men, but not the sort to appreciate your work. In fact I have

long thought that *too much* systematic work and description somehow blunts the faculties. The general public appreciates a good dose of reasoning, or generalisation, with new and curious remarks on habits, final causes, &c. &c., far more than do the regular naturalists.

And he wrote to Hooker in 1853:

How few generalizers there are among systematists; I really suspect there is something absolutely opposed to each other and hostile in the two frames of mind required for systematising and reasoning on large collections of facts.

In the first years after the publication of *Origin of Species*, the only public notice taken of the Darwinian theories by the London Entomological Society was this facetious comment made by H. T. Stainton to the group in 1860 when he noted the crowding in the meeting room and said that, if it continued,

many of our members would succumb in this new struggle for existence, and those entomologists best fitted to breathe our vitiated atmosphere would have advantages over their fellows, and would become the favored race; and supposing they transmitted these qualifications to their descendants, it might happen, in the course of a few hundred years, that a race of entomologists would be produced who would positively feel uncomfortable unless in rooms crowded to suffocation.

But during the first years after the *Origin* was published, there was always a small group of pro-Darwin entomologists such as H. W. Bates, A. R. Wallace, John Lubbock, Roland Trimen, Raphael Meldola, and a scattering of non-British entomologists. These men were active members and held high offices in the entomological world, and their efforts to convince their associates of the truth of Darwinism gradually prevailed, as noted above.

Darwin's direct contributions to entomology during his lifetime were outstanding. But his grandest achievement was the discovery and demonstration of the importance of natural selection and his convincing marshalling of the facts, which led to the general acceptance of the reality of organic evolution; this has had a profound impact on the direction of entomological research.

Although a curiously large number of insect taxonomists continue to practice in a manner little, if at all, different from their pre-Darwinian predecessors, specialists in most other areas of insect biology, as well as many taxonomists, work with far more sophistication than did their progenitors. In most instances there is a clear Darwinian thread through their investigations, leading to a comparative point of view, which is no less important than the spread of the experimental method and improved techniques.

While Darwinian theory has had a major effect on entomology, the reverse is also true. Perhaps because of the great number of entomologists and of insect species, entomology in the broad sense has made a larger contribution than any other field to the elaboration of the evolutionary idea since

Darwin's time. For example, the greatest obstacle to acceptance of the evolutionary idea by many nineteenth century biologists was the almost total ignorance of what we now call "classical genetics." This lacuna no longer exists, and *Drosophila* (an insect genus!) has stood beyond all other organisms in importance for working out the mechanisms of heredity. Many other areas of insect biology have provided major sectors of the formulation of modern evolutionary theory. Especially notable has been entomological work on population genetics, adaptive coloration and mimicry, cytotaxonomy, niche ecology, paleontology, comparative behavior, biology of sex, environmental physiology, and biogeography.

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# INSECT NUTRITION[1]

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Insect nutrition concerns more than mere dietetics. In its broadest sense it involves many metabolic processes. It is a bond between physiologic and ecologic phenomena associated with natural selection and competition for food (48). Research on it determines essential nutrients and illuminates metabolic pathways, genetic mechanisms, and comparative biochemistry.

With few exceptions, this is a review of work done since the last general review of the subject (92) and includes some information reported later in more specific reviews (40, 66, 86).

Some terms used throughout this paper should be defined to avoid confusion. According to Beck (5), the term "nutritional requirements" should refer only to the chemical factors essential to the adequacy of the ingested diet; "chemical feeding requirements" refers to the chemical factors important to normal feeding behavior; and "physical feeding requirements" refers to the insect's requirements as far as dietary texture, position, light intensities, and other physical factors influencing feeding behavior are concerned. These terms are used in this review as Beck defined them. The terms "axenic," the rearing of only one or more individuals of a single species on a non-living medium, and "xenic," the rearing of an organism in association with one or more unknown species of organisms, are used as Dougherty (33) defined them. In this paper, a "chemically defined diet" is one that Dougherty (33) terms either "holidic," "pertaining to media whose intended constituents, other than purified inert materials, have exactly known chemical structure before compounding"; or "meridic," "pertaining to media composed of a holidic base to which is added at least one substance or preparation of unknown structure or of uncertain purity." Generic and specific names are those in current usage.

## Methods of Study

Nutritional data come from a spectrum of works ranging from those on natural foodstuffs to those for precisely determining nutritional requirements on chemically defined diets, often axenically. Very useful techniques with labelled isotopes (77) and with antimetabolites (90, 127) have been demonstrated, but feeding tests are most commonly used. Though work on natural foodstuffs may be heuristic, it is severely limited by lack of sufficiently detailed food analyses. It is seldom precise enough, and it may even fail to distinguish between nutritional and non-nutritional responses. Techniques on excised plants have serious inherent sources of error (5).

[1] The survey of literature pertaining to this review was concluded in February, 1960.

To determine nutritional requirements, one should know the chemical composition of the diet and be able to vary its composition precisely so that effects on the insect may be related accordingly. Diets should enable normal development over many generations, and microorganisms must be eliminated (40). It may be important to consider the source and method of maintenance of the test insect (82). I would add that experiments should be designed to provide quantitative data permitting statistical analyses; for, according to Snedecor (137), it is only by a combination of appropriate design, skillful conduct of the experiment, and suitable statistical methods that the investigator is assured of reliable evidence upon which to base his decisions. In this subject, many authors simply state a value with no indication of the number of determinations or of insects involved. Though growth responses are typically variable, no measure of variation is given.

Axenic techniques are necessary in standardizing test animals and in controlling their environment (113). Axenic techniques separate the specific metabolic needs of a species from any possible host-symbiote relationship (33). Fraenkel (37) stated that sterile methods are essential where the food is wet and, where necessary, to prevent change in the composition of the food, but, though there is no doubt about the nutritional importance of symbiotic microorganisms, intestinal microorganisms are of doubtful importance in many species of insects. He pointed out that nutritional requirements of many stored-product insects were determined by xenic methods. Nevertheless, in the work by Pant & Fraenkel (107), for example, microbial factors had to be eliminated before the nutritional requirements of the insects could be determined on dry foods.

Unnatural food and feeding conditions, however, may not be conducive to optimum nutrition. They must first be chemically and physically attractive to incite satisfactory feeding responses. For example, the fastidious species *Bombyx mori* (Linnaeus) was induced to feed on nutritionally inert agar impregnated with three chemical attractants from mulberry leaves (52). In another work (123), failure to fulfill chemical and physical feeding requirements resulted in suboptimal growth of test insects on nutritious diets. During larval development, the physical properties of wet media may change and adversely affect the insects (131). Osmotic pressures of some media have prevented inclusion of sufficient concentrations of nutrients for optimal nutrition (96, 129). A constant diet of uniform composition may not be best. For example, in adult females of *Calliphora vicina* Robineau-Desvoidy [= *C. erythrocephala* (Meigen)], ingestion of protein was relatively high and that of carbohydrate low during the early stages of egg growth, but, during egg formation, ingestion of carbohydrate was high and that of protein low (138). In other work, requirements were shown to change with larval development (5). Young larvae may be the most susceptible to deficiencies (71). Problems of creating acceptable media were discussed by Friend (39), and a pithy paper by Lipke (91) is instructive.

More or less chemically defined diets have been developed for many in-

sects, usually in conjunction with axenic techniques. At least nine of the order Diptera are included (18, 42, 43, 68, 70, 96, 120, 125, 130). Lepidoptera are represented by three species (9, 74, 142). Coleoptera are represented, though not with axenic techniques (87, 99, 108). Orthoptera are represented by *Blattella germanica* (Linnaeus) xenically (48) and aseptically with inherent bacteroids (65). Heuristic work (123, 124) on two species of Hemiptera is noteworthy. Most of these insects have relatively simple feeding habits and requirements.

Insects are the only invertebrate Metazoa for which such media and rearing techniques have been developed (33). Geneticists were foremost in this achievement with *Drosophila melanogaster* Meigen (122). No metazoan has yet been propagated indefinitely on media of exactly known composition (33). However, *Pectinophora gossypiella* (Saunders) (142) and *D. melanogaster* (122) were reared through more than one generation on diets that were highly defined, but that contained agar and other possible sources of undefined nutrients. *B. germanica* was reared xenically through several generations on deficient diets of known composition to determine cumulative effects of long-term deficiencies (48).

### Qualitative and Quantitative Nutritional Requirements

Much work is done on qualitative requirements, which generally differ very little among the insects thus far investigated (66). Possibly such similarities have been overemphasized, since a few differences exist that indicate probable important differences at a metabolic level (67). Some of these nutritional differences are mentioned in various sections of this paper. More work is needed to determine whether they are specific differences or have greater significance. Except to point out a few examples, especially of something new or unusual, qualitative nutritional requirements need not be fully discussed in this paper. Quantitative nutritional requirements of insects seem to be complex and possibly are of greater significance than the qualitative requirements. In either case, requirements have usually been determined for only one generation of nutrition, though they may depend on nutritive reserves. The importance of the reserves that are carried through the egg were discussed (30, 48, 67, 122). Nutritive reserves also play roles in the nutrition of adult mosquitoes (139, 140) and of black flies (Simuliidae) (26).

Recent developments in qualitiative work may be briefly reviewed. The nutritional value of proteins, as shown with proteins or equivalent amino acid mixtures, varies for growth (66, 143) and also for reproduction (84, 102). Egg albumen probably has superior qualities for fast growing animals like insects (48). The amino acids needed for growth were determined in a number of insects (18, 27, 28, 41, 96, 108, 130, 143, and others). Generally insects, like higher animals, need the well-known 10 essential amino acids. These 10 were needed for reproduction in *Aedes aegypti* (Linnaeus) (31, 130). These results with *A. aegypti* females should be applied with caution to other insects because of physiological differences (30). There are some exceptions

concerning amino acid requirements for growth. For example, proline, usually considered dispensable, is essential in *Phormia regina* (Meigen) (96), though it is synthesized by this insect (77). *P. regina* has alternative requirements, as it metabolically "prefers" certain amino acids to others (19, 64). Usually dispensable amino acids must supplement essential ones for optimal nutrition of insects (83, 108, 143). Though not essential, iodoproteins and iodoamino acids promoted growth and development of *Corcyra cephalonica* (Stainton) (100).

Utilization of carbohydrates varies with the species and depends on abilities to digest poly- and oligosaccharides to diffusible, absorptive forms and on the degree of absorption (110). The nutritive values of a great number of carbohydrates were rated for growth (62, 109), longevity (45, 104, 147), and fecundity (32). *Musca domestica* Linnaeus is one insect that needs no carbohydrate for growth (14). The presence of certain sugars in the larval diet of *D. melanogaster* favored their utilization by the adult; utilization in adults may differ with sex (104).

Qualitative vitamin requirements of a number of insects were determined (42, 70, 81, 106, 126, and others). Several vitamins increased fecundity and fertility in adult tephridids (51), but vitamins had no such effect on adults of *A. aegypti* (130). Five or six B vitamins are commonly needed for growth and development, but requirements for others vary with the insect. For example $B_{12}$, though active in purine synthesis (119), is not required by some insects (120, 126), but lack of it arrested growth of *B. germanica* during the second generation on the diet (48). Inositol was needed by two species of cockroaches (34, 48), though it is generally considered dispensable in insect diets. Similarly, ascorbic acid was found to be necessary for growth of *Schistocerca gregaria* (Forskål) (25). It would seem that carnitine, or vitamin $B_T$, was a larval requirement of only several Tenebrionidae and that nucleic acids were needed only by dipterous larvae were it not for reports of beneficial effects of $B_T$ on *A. aegypti* (130) and of ribonucleic acid (RNA) on the beetle *Sciobius granosus* Fåhraeus (117). The requirement for RNA in *D. melanogaster* is primarily one for adenylic acid, essentially for adenine (121). A need for a fatty acid, linoleic, was shown in two lepidopterans (141, 144) and possibly in *B. germanica* (15; cf. 48).

The effects of various sterols on growth of several insects confirm that cholesterol or similar sterols are needed by insects (54, 112, 145, and others). Cholesterol deficiency in *M. domestica* larvae subsequently reduced egg hatchability (98).

Mineral requirements of insects probably remain the most neglected area of insect nutrition. The essential elements for *D. melanogaster* include potassium, phosphorus, magnesium, and sodium, but not calcium, except possibly in trace amounts (120). Manganese, zinc, and copper are probably essential in *B. germanica* (48). Beneficial effects of several mineral salts on growth of *B. germanica* and on the transmission of bacteroids were shown by Brooks (15). The expression of a carnitine deficiency in *Tenebrio molitor* Linnaeus de-

pended on zinc and potassium in the diet (36). Other effects of zinc and copper were shown in *C. cephalonica* (132, 133). The effects of water on the nutrition of certain insects were shown (81, 97, 148). Several unidentified factors may also be needed in some insects (11, 44, 134).

For optimal nutrition the insect must obtain quantities of nutrients sufficient for its needs. Fecundity in *Dacus dorsalis* Hendel increased with increase of dietary proteins (51). Nutrition of *D. melanogaster* depended on a satisfactory level of dietary protein; too little prolonged growth, and too much upset metabolism (120). This was more or less so for fructose, choline, and certain other nutrients (120). Sugar levels determined growth rates in other insects (53, 68, 128). The nutritive efficiency of a M/5 solution of glucose was equal to that of a M/10 solution of sucrose in *D. melanogaster* (104). The minimum dietary levels of different vitamins needed for good nutrition were determined for *D. melanogaster* (120) and *C. vicina* (125). Generally, amounts in excess of these minima had little or no effect. Choline was an exception in *D. melanogaster*, as excess amounts were detrimental (120). Forgash & Moore (35) clearly showed that inositol is required by *Periplaneta americana* (Linnaeus); that, as the dietary level of inositol was increased, there was a corresponding increase in survival and rate of growth and development; and that the maximum response occurred with 40 to 80 mg. per kg. of diet. Growth rates of insects were proportional to the supply of cholesterol up to a limit, but excessive amounts were not very injurious (89, 120). Levels of RNA greater than half that needed for optimal growth of *Agria* [= *Pseudosarcophaga*] *affinis* (Fallén) resulted in fewer adults (69). The quantities of a salt mixture and of various mineral elements needed for optimal nutrition of *Tribolium confusum* Duval were determined (72, 73). Growth may depend on the water content of a food (81). Sang (120, 122) stated that comparisons of minimum supplies of nutrients necessary for nutrition of species having different feeding habits are of limited value. Estimates of minimum requirements give no direct measure of the quantities of each nutrient needed by a larva; they define only the relationships between particular requirements, and a strain that has the same absolute requirements but feeds faster than another would regularly show lower minimum requirements, and vice versa (120). A satisfactory uniform method of stating quantitative requirements is needed (67, 120). Sang (120) determined the amount of each vitamin needed to produce one gram of *D. melanogaster* pupae, and proposed this criterion as a suitable means of circumventing the problem of comparing requirements of insects that have different rates of intake and different times spent feeding (122).

Quantitative requirements depend on the proportions of nutrients. It is generally recognized that some balance is needed between the main classes of nutrients, proteins, carbohydrates, lipids, and so forth for good nutrition. The ratio of sucrose to protein is 7:1 in diets for laying females of *M. domestica* and 16:1 for males and non-laying females (49). Satisfactory proportions are necessary within and between classes of nutrients, especially amino acids

(14, 44, 48, 60, 67, 83, 120, 122). The amounts of the essential amino acids needed by *Apis mellifera* Linnaeus [= *A. mellifica* Linnaeus], expressed as ratios with the amount of tryptophan required, were found to be very similar to those for other animals compared on a similar basis (29). Small differences in the proportions of 19 amino acids had gross effects on growth of different insects (41, 67), and mixtures of these amino acids that are suitable for one species may be unsatisfactory for others (67, 143). Sang (122) stated that it would be possible to select from two different insects strains that would have the same quantitative vitamin requirements. He described a number of circumstances affecting the nutritional requirements of an insect that show that quantitative requirements are relative. For example, the amounts of various vitamins needed by *D. melanogaster* depended on protein levels; the balance between protein and sugar in the diet may be altered and so affect vitamin needs; folic acid could be partly replaced by purine, serine, or other substances, or by any combination of these (122). There can be a multiplicity of balances between nutrients, each resulting in a diet equally as good as another for optimal nutrition (48, 122). And there can be injurious imbalances. Thus, quantitative nutritional requirements of insects become important at a level that formerly may not have been sufficiently appreciated (67).

### Genetic Bases of Nutritional Needs

According to Hinton & Dunlap (61), there is a genetic basis for nutritional requirements, since metabolites may become requirements, depending on genetic constitution. Nutritional requirements were found to differ within certain species of insects (24, 82, 84, 85). The most fundamental work is on *D. melanogaster*. Strains were demonstrated in which many variations in needs occur for RNA, its components, and several amino acids (60, 61). In some, the sex chromosome was involved in adenine synthesis, so that RNA was required; another strain gradually changed its adenine requirement: originally with RNA in the larval diet no adults eclosed, now about half do (60). There were no remarkable differences in nutritional requirements between several "standard" strains (122).

Sang (119) determined differences in the nutritional requirements of *D. melanogaster* in relation to heterosis. He found, for example, that the minimum requirement for nicotinic acid in a hybrid was intermediate between those of its parental strains. As growth and development of hybrids are superior in many ways to those of parental strains, he concluded that hybrids, which use more, or make better use of, vitamins, have more effective enzyme systems and, hence, are biochemically more efficient than their parental strains.

According to Sang (122), differences between the needs of *D. melanogaster* provide a selective advantage and indicate the likelihood of adaptation to nutritional environments within and between species. Nutritional differences resulting from evolution may be most apparent at the level of metabolism;

hence, differences in the utilization of substances and the peculiarities of adaptation to particular environments are significant (122).

### Relation of Nutritional Needs to Metabolism

At low temperatures, *A. mellifera* increased its metabolic rate, heat production, and food consumption (38). At suboptimum temperatures, carbohydrates had beneficial effects on reproduction of blood-fed females of *Anopheles maculipennis* Meigen but no apparent effect at optimal temperatures (55). In *D. melanogaster*, minimum vitamin requirements depended primarily on metabolic rate and not on size (120). Of the vitamins required, only thiamine and riboflavin, which are involved in energy metabolism, were unaffected by protein supplies (122).

Because nutritional requirements depend on metabolic processes, determination of nutritional needs can lead to biochemical explanations of the capabilities for synthesis and of other processes. Sang (122) stated that nutritional techniques are not as blunt an instrument for doing this as is sometimes supposed. Hinton (60) listed the substances that are essential in *D. melanogaster;* those that are not absolute requirements and are probably synthesized, and those that may be substituted for others in nutrition. Feeding tests demonstrated that different transulfuration mechanisms exist among insects. For example, *B. germanica* needed no dietary cystine or methionine in the presence of a supply of inorganic sulphates (56). In fact, it needs no supply of organic sulphur (48). *P. regina*, on the other hand, needs either cystine or methionine, but not both (64). Usually, of the two, only methionine is apparently needed (57). Feeding tests with intermediary metabolites established the existence of a phenylalanine cycle in *B. germanica*, thereby explaining why neither phenylalanine nor tyrosine is essential when sufficient tryptophan is fed, and why phenylalanine and tyrosine are nutritionally equivalent in the roach (48). Possibly no such cycle occurs in *A. aegypti*, according to Gordon (48), because tyrosine cannot replace phenylalanine and both phenylalanine and tryptophan are essential for growth of this mosquito (130). Earlier work (46) showed that neither tyrosine nor phenylalanine alone was essential, though omission of both in the presence of tryptophan prevented growth of *A. aegypti*. On the basis of feeding tests, Hinton (59) concluded that *D. melanogaster* has no ornithine cycle, since arginine was essential, citrulline only partly replaced it, and ornithine had no effect. Ornithine was not an efficient precursor of arginine in *B. germanica* (48). Nutritional techniques showed relationships between glycine, serine, and folic acid and between protein supply, glutamic acid, and nicotinic acid in *D. melanogaster* (122).

Feeding techniques were used to determine the utilization of purines and pyrimidines (58, 121). The requirement for RNA in *D. melanogaster* was primarily one for adenylic acid, but other substances may promote growth; some, together with adenylic acid, were equivalent to RNA. The pyrimidines

and their nucleosides and nucleotides were not utilized alone (120). Sang's (121) results differ in but a few respects from those of Hinton (58). RNA determined the need for folic acid, which acts in the conversion of uridine to thymidine (122). Possibly some insects may utilize nucleic acids differently from others. For example, *D. melanogaster* (121) and *A. affinis* (69) develop without dietary nucleic acids but grow faster with RNA. However, *A. affinis* can use deoxyribonucleic acid (DNA) (69), but *D. melanogaster* cannot (118, 146). Feeding DNA overcame the detrimental action of a folic acid antagonist, aminopterin, in *D. melanogaster* (47). Both RNA and DNA in the diet overcame the inhibition of growth in *C. cephalonica*, and $B_{12}$ overcame mortality caused by zinc toxicity (133). The behavior of $B_{12}$ was explained on the basis of its possible involvement in nucleic acid synthesis in the larvae, particularly in view of the influence of nucleic acids on zinc toxicity.

Sterol metabolism and insect nutrition were discussed by several workers (8, 12, 17). Some (20, 89) fed different insects various sterols, and, from these works, we may conclude that utilizable sterols have certain identified molecular configurations in common. Feeding tests by Noland (103) showed that *B. germanica* could utilize ergosterol. Gordon (48) could not confirm this satisfactorily. With diets like those used by Noland, however, Clark & Bloch (23) found that *B. germanica* grew with ergosterol as the sole source of sterols, and, by subsequent biochemical techniques, they found that ergosterol was converted into a cholestane derivative. They (22) found that ergosterol was inactive in *Dermestes maculatus* DeGeer [= *D. vulpinus* Fabricius], though growth was promoted by certain other sterols in diets containing subminimal quantities of cholesterol. Evidently *D. maculatus* lacked the necessary mechanisms for modifying ergosterol (23). Feeding sterol precursors, such as mevalonic acid, squalene, and lanosterol, neither replaced nor spared cholesterol needs in *D. maculatus* (21). This suggested to the authors (21) that the pathways of cholesterol biogenesis are multiple-blocked in this insect and not just interrupted at the squalene stage, as previously reported (13). Levinson & Bergmann (89) observed that the kind and number of sterols used by insects that feed on both animal and plant material seem to be intermediate between those used by wholly phytophagous and wholly zoophagous species.

This discussion does not exhaust all the cases in which nutritional techniques probed metabolic processes. Certainly, purely biochemical investigation was necessary in many instances in order to advance work to our present understanding of metabolic processes. Limitations of space and subject necessitate omission of these. The roles played by many nutritionally important substances in various aspects of insect biochemistry were discussed recently (88, 114).

## Ecological Significance of Nutritional Needs

There is evidence that intricate relations exist between insects and their food plants. Sex ratio, reproduction, and size and survival of the female as

compared with the male varied in several species of hymenopterous parasites with the food plant of their host, *Aonidiella aurantii* (Maskell) (136). Populations of *Aphis pomi* DeGeer were decreased on leaves in which metabolic changes in the RNA/DNA ratio were induced experimentally: similar metabolic changes occur during natural development and ageing of leaves (80). *Pieris brassicae* (Linnaeus) was affected in various ways on leaves deficient in nitrogen, phosphorus, potassium, and iron (1). High water content of rice plants was generally associated with fast rates of development and reproduction of several species of rice weevils (101). Nutrition may not be primarily responsible for these effects in all cases, since feeding behavior and other factors may be implicated. For example, *Aphis fabae* Scopoli decreased feeding activities and larviposition rates and produced most alate forms on water-deficient leaves (79). Beck (4) claimed that, though *Pyrausta nubilalis* (Hübner) larvae fed mostly on corn tissues containing the highest sugar levels, the high mortality on corn leaves was not caused by nutritional deficiencies but by toxic substances, one of which had a biological activity inversely proportional to the sugar content (6, 10). Feeding behavior of the young larvae was correlated to some extent with molecular weight and water solubility of a number of amino acids (7).

The effects of various chemicals on plants may affect pest insects. For example, copper that was supplied to growing wheat and soybean improved the nutritional value of their products for *T. confusum* (20). The rate of supply of nitrogen to chrysanthemum plants and its concentration in the leaves correlated well with the extent of damage by *Liriomyza trifolii* (Burgess) [=*L. pusilla* auct.] (149). Ishii & Hirano (75, 76) related superior growth of *Chilo suppressalis* (Walker) to the high nitrogen content of rice plants cultured in nitrogen-rich soils and solutions, but they found that phosphorus fertilizers generally had no significant effect (63). Growth of the aphid *Acyrthosiphon pisum* (Harris) improved on pea cuttings that were maintained in solutions of various substances that normally occur in aphid blood, such as asparagine and glutamine, but amino acids alone had no effect (93). The resistance of aphids to insecticides differed with the host plant on which they were reared, and the differences were considered to be attributable to nutrition (111). Work on mites, *Tetranychus telarius* (Linnaeus) and *Tetranychus tumidus* Banks, indicated that soil insecticides may change the nutritional value of the host plants, since the mites responded accordingly when the potassium, phosphorus, and nitrogen contents of the plants were altered (115). Some herbicides applied to plants increased reproduction in feeding aphids; others increased mortality or reduced fecundity (95). Rodriguez (116) concluded that several kinds of phytophagous pests benefit from an oversupply of nitrogen, and that the host plant can be affected by the addition of fertilizers, insecticidal and fungicidal sprays, and soil insecticides.

The important question arises: is varietal resistance in plants attributable to differences in content of substances nutritionally important to in-

sects? Developments in research on resistance of plants to insects have been reviewed fully (2, 5, 40, 92, 105). Auclair (2) allows that adverse effects on insect development in some cases may be caused by the unsatisfactory nutritional value of the food plant. Beck (5) found little experimental evidence up to 1956 to support such a hypothesis, though nutritive factors contributing to host-plant adequacy had been identified. In some more recent work, nutritional factors seem to be implicated in varietal resistance. For example, fertility in *Leptinotarsa decemlineata* (Say), which varied with both species and variety of host plant, was affected on potatoes more by the lecithin content of the leaves than by the carbon-nitrogen ratio (50). Varieties of peas most susceptible to the aphid *A. pisum* contained more nitrogen and less sugar than did resistant varieties (94); it was concluded that, consequently, the aphids probably encountered great nutritional differences between susceptible and resistant pea plants. Susceptible varieties generally contained the highest concentration of free and total amino acids (3). It was suggested that the lower concentrations of amino acids in the resistant varieties reduce the rate of growth and reproduction of the aphids and so contribute to the resistance of these varieties. Auclair (2) pointed out the need for information, especially on the quantitative nutritional requirements of phytophagous insects, and for work with phytophagous insects in which unnatural diets, particularly chemically defined diets, are used to investigate the problem of resistance of plants. Some workers consider the efficiency with which digested food supplies materials for energy and for body tissues to be important. For example, one gram of wheat meal produced 0.214 gram of adult of *Lasioderma serricorne* (Fabricius) (16). Conversion of wheat plant into body tissues of *Melanoplus bilituratus* (Walker) was intermediate in efficiency between those of the oat plant and of western wheat grass (135). The consumption, excretion, and increase in body tissue of dry matter and nitrogen in *Agrotis orthogonia* Morrison fed on two varieties of wheat differed in some ways (78). Gordon (48) pointed out that "high food efficiency" is biologically desirable, since it increases the number of organisms that can be produced on a given amount of food. Hence, selection pressure occurs favoring genotypes than can utilize a given food source with maximum efficiency. Therefore, he states, the ecologically significant question is not "what is an optimal diet for this animal?" but "what is the most deficient and unbalanced diet that the animal can tolerate without drastic reduction of its rate of growth and reproduction?"

In almost all work to date on this problem, very little, if anything, was known about the actual nutritional requirements of the insect involved or about the chemical composition of the plant. The best exception to this is the previously cited work by Beck and his co-workers, in which no nutritional factor was primarily responsible for resistance in the corn plant. This result, however, does not yet allow generalizations to be made concerning resistance of plants and insect nutrition. What seems necessary is more work in which sufficiently detailed qualitative and quantitative analyses of the

chemical compositions of host plants are related to the qualitative and quantitative requirements of the insects concerned. Painter (105) stated that not enough emphasis has been placed on the use of resistant varieties, particularly resistant and susceptible isogenic pairs, as tools in the study of insect nutrition. This problem, therefore, cannot be conclusively solved until much more critical investigation is done.

## Concluding Remarks

A reasonably adequate insight into insect nutrition has been gained during the last decade or so. But this insight should be extended to include insects that differ widely in taxonomic position and in feeding habits. Very little is yet known about the nutrition of insects of some orders, of highly specialized parasitic species, or of others that feed on only one or a few foods.

Possibly the most important development in the subject during the last four or five years is the increasing understanding of the interrelations determining the nutritional needs of an insect. In particular, recent works showed precisely the relations of many nutritional needs to metabolic processes in insects and demonstrated the genetic bases for some needs. Moreover, they helped gain an understanding of genetic and metabolic mechanisms. Nutritional requirements for growth, development, or reproduction may depend on reserves of substances stored in the body or egg sufficient for the needs of the insect for part or all of one generation. Hence, when deficiency symptoms first become apparent, at pupation, for example, can we say that a substance is required only for pupation if nutritive reserves are sufficient for nutrition up to this stage? Nutritional requirements cannot be completely understood until work is done on insects in which such reserves are depleted. Gordon's (48) recent work with *B. germanica* is possibly the first step taken in this direction with insects.

It has not been determined whether resistance of plants depends to any significant degree on nutritional factors important to insects. Possibly the techniques used were not always sufficiently critical. If nutritional control of phytophagous insects is possible, it seems most probable that it will arise from feeding on unsatisfactory proportions of plant substances so that the insects will be inefficient in utilizing the food. Possibly plant breeders can develop varieties of plants unsuitable as food for the insects concerned, as was suggested (41). We have seen, however, that nutritional requirements may vary within a species, and this gives insects selective advantage whereby they may readily adapt to new nutritional environments.

Therefore, insect nutrition work may serve a wide area of biological research. Insects are the only invertebrate Metazoa that have been reared axenically on chemically defined diets (33). Consequently, insects offer especially favorable material for elucidating general principles of animal nutrition and related subjects.

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# NUTRITIONAL FACTORS IN INSECT RESISTANCE TO CHEMICALS[1,2]

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## Present Status of the Insecticide Resistance Problem

The number of insect species or populations resistant to one or more of the synthetic organic insecticides has increased every year since 1947, and there is as yet no indication that this trend can be halted or reversed. Extensive research on insecticide-resistant strains has shown that they probably arise by selection and recombination of "R genes," which are present at low frequencies in the original population (23, 115, 161), and that these genes confer high resistance primarily by producing enzymes that efficiently destroy chemically similar insecticides (24, 105, 122a). Based on this knowledge of the mechanism of resistance, three tentative solutions have been proposed: (*a*) Lower selection pressure (26, 71): this requires that a large fraction of the S insects in the original population be allowed to survive (by using low insecticide dosages or by treating limited areas) and assumes that their greater viability will compensate for their higher mortality under insecticide treatment, so that the frequency of R genes in the population will not increase. (*b*) Intermittent selection: this requires that any one insecticide be used only for a limited time, followed by intervals of reliance on "natural controls" (integrated control) or on a different insecticide (alternation) (147, 159). Intermittent selection presupposes that many S insects have survived and that R insects will be more susceptible to "natural controls" or to the alternate insecticide. If this happens, the increase in frequency of R genes for the first insecticide during its period of use will be compensated by a decrease during the period of disuse. (*c*) Combinations of synergistic insecticides: the ideal combination would be two insecticides showing negative cross-resistance so that an increase in resistance to one insecticide would cause an increase in susceptibility to the other (24). The combination would therefore exert no selection pressure for resistance to either constituent.

The difficulty in all these tentative solutions of the resistance problem is that the negative cross-resistance on which they rely is unlikely to be very effective. The presence of R genes for one insecticide is usually not a severe

[1] The survey of the literature pertaining to this review was concluded in June, 1960.

[2] Throughout this chapter the symbols R and S are used to designate insecticide-resistant and -susceptible insects and their genes, enzymes, and other characteristics. The term "cross-resistance" refers to a change in resistance of R insects to some deleterious factor other than the insecticide to which they have become resistant. Cross-resistance is "positive" if resistance to the other factor has been augmented, "negative" if it has been lowered, and "zero" if it is unchanged.

handicap to survival under natural control (17, 178), and the frequency of R genes does not decline rapidly to zero when all insecticide pressure is removed (82). When a second insecticide is used, the presence of R genes for the first is often advantageous, because there is actual or latent positive cross-resistance (52, 81). The many vigorous multiresistant strains now known prove that R genes for many insecticides can coexist in one individual, without necessarily rendering it very susceptible to the action of natural controls. It seems probable that any selection by insecticides will cause an increase in the frequency of R genes that is proportionate to the reduction in the total population and somewhat cumulative (186). This, in time, must cause some decrease in the effectiveness of insecticides in insect control.

## Thenetic (Non-Hereditary) Effects of Nutrition on Insecticide Tolerance

The tolerance of a susceptible population is determined by many physiological variables. Qualitatively, these are controlled by a complex polygenic system, each element of which influences to some extent the uptake, penetration, transport, distribution, toxic action, detoxication, and excretion of the insecticide. Quantitatively, they may also be influenced by environmental factors, such as temperature or nutrients. It is difficult to isolate these interdependent variables experimentally and to decide what contribution each makes to the over-all tolerance, except when one is the dominant factor. For example, it has been possible to show that the high tolerance of *Melanoplus femur-rubrum* (DeGeer) to DDT is largely attributable to its impermeable cuticle (165); the high tolerance of *Musca domestica* Linnaeus to Sevin® (1-naphthyl methylcarbamate), to a Sevin-detoxicating enzyme (47); and the high negative temperature coefficient of DDT-tolerance in *Periplaneta americana* (Linnaeus), to the enhanced sensitivity of its nerve fibers to DDT at low temperatures (194).

There are few examples of the influence of nutrition on tolerance. *Macrosiphum pisi* (Harris) [=*Acyrthosiphon pisum* (Harris)] reared on *Vicia faba* are about 1.5 times more tolerant to rotenone than aphids reared on *Trifolium pratense* (141). *Myzus persicae* (Sulzer) reared on turnip (*Brassica campestris*) or lettuce (*Lactuca sativa*) are three times more resistant to nicotine than aphids reared on nasturtium (*Tropaeolum* sp.) (145). Adult *Musca domestica vicina* Macquart are somewhat more resistant to DDT when fed on goat's milk than on cow's milk or other diets (126). On the other hand, larvae of *Aedes aegypti* (Linnaeus) reared on milk are somewhat more susceptible to DDT than those reared on wheat flour (45). A diet abnormally rich in protein decreases the resistance of *P. americana* to DDT about twofold (100). All these effects are minor and difficult to interpret. Even if it could be shown that a diet that causes higher tolerance also causes larger body weight or higher fat content, one could never be certain that the increased body weight or fat is the direct cause. Some other, unknown factor may be responsible.

An interesting nutritional effect has been observed in a dieldrin-R strain

of *M. domestica* (21). If larvae of this strain are reared on a dieldrin-free medium, the $LD_{50}$ of dieldrin to the adult females is about 2 μg./fly and is only slightly increased by the addition of milk fat to the glucose-water diet of the adult flies. If the larvae are reared in a medium containing a high (but sublethal) concentration of dieldrin, the adult females contain over 5 μg./fly of dieldrin, but the $LD_{50}$ of dieldrin applied topically is 4 μg./fly if they are fed glucose-water and 17 μg./fly if milk fat is also fed. The total fat content of the flies is the same as, and in fact is not higher than, that of a dieldrin-S strain. This work suggests that nutritional effects on insecticide tolerance can be more striking if the insecticide is present (at sublethal levels) in the insect body during the feeding period. Adaptive changes that increase tolerance of an individual insect to a specific toxicant are more likely to occur and to be influenced by diet if the toxicant is chronically present. The changes may not be as large as the eightfold increase in the $LD_{50}$ value of dieldrin suggests, because, at dosages above 1 μg./fly, the rate of penetration of insecticides does not increase in direct ratio to the quantity applied. Whenever a toxicant is relatively ineffective, the ratio of quantity penetrating/quantity applied decreases as the applied dose increases; this is one of the factors that contribute to the extremely low slopes of log dosage-probit lines in highly resistant strains (72).

Field studies often show a significant increase in the resistance of insect populations with seasonal changes (2, 84), and some of this increase may be attributed to the changing nutritive value during growth and maturation of the crop plants and also to adaptive changes in the insects growing in the presence of sublethal levels of insecticides. Lygus bugs from an alfalfa hay field not treated with insecticide were only one-fifth as resistant to DDT as bugs from an alfalfa seed field repeatedly treated with insecticide (2); in this case, however, the changes in the mortality curves suggest that susceptible insects were killed early in the season and the survivors gave rise to a true low-resistance or vigor-tolerant strain. In the next season, the overwintering individuals of this strain would be mixed with susceptible insects from nearby untreated areas to give a highly variable early-season strain, which is then reselected by the early insecticide treatment to give the less variable and more tolerant late-season strain. The tolerance will be influenced by environmental factors, but the field data suggest that this is a minor effect.

As knowledge of nutrition has increased, it has become clear that, as a result of natural selection, all potential food sources may have become to some extent imbalanced (60, 152). They have deficiencies or excesses that depress the growth rate of the organisms that eat them to a level considerably below the maximum possible growth rate. The plant sap on which aphids feed has quite low levels of some amino acids (8, 119), and some differences in blood amino acids of *Prodenia eridania* (Cramer) raised on kale and on potato have been noted (75). If a nutrient is absorbed in excess of the requirement, it must be destroyed, and the rate of growth will be limited by the rate of destruction; if a nutrient is deficient, this, in effect, means that all others are

present in excess and must be unabsorbed, destroyed, or excreted. Different diets cause different degrees of "overloading" of the destructive systems present and may cause a selective increase in reserves of some adaptive enzymes (9, 51). Such effects will usually be minor and not of sufficient practical importance to merit large-scale investigation. It is the long-term genetic selection effects of diet on the tolerance and potential resistance of insect species that merit review and further research.

## Genetic Effects of Nutrition on Insecticide Tolerance

The thesis of this review is that the R genes that by selection and recombination under insecticide pressure give rise to R strains are alleles of common genes, the normal function of which is metabolism of biochemicals present in the normal insect dietary. In a sense this is obvious, but the primary and dominant role of nutrition in the determination of the biochemical activities and potentialities of living organisms is rarely given enough emphasis.

The complex biochemical interaction in which insects play a role can best be understood by considering the selective forces that determine the biochemical composition of an edible plant. The primary forces are physico-inorganic (soil, water, light, etc.), and the essential biochemical core of the plant consists of systems for the intake of raw materials and energy and their utilization to synthesize more protoplasm. Selection acts to increase the efficiency of these processes, and, since there are probably only a few relatively similar, highly efficient systems, selection tends to maintain a high degree of similarity in the fundamental biochemistry of all plants.

The secondary selective forces arise from competition with other plants. These drive plants into restricted ecological niches (shady and moist, dry and sunny, etc.) to which they are better adapted than most of their competitors. Although each niche probably selects variations in the primary biochemistry (such as the ratio of chlorophyll A to chlorophyll B), much of the adaptation must be secondary (such as efficient wax coating of leaves in xerophytes).

The tertiary forces consist of the infinite variety of assaults on the plant by microorganisms, insects, and herbivorous vertebrates. These heterotrophs are selective agents for resistance to themselves, which can be of two main kinds: unassailibility (mechanisms that hinder feeding; e.g., the impenetrable seed-coats of some angiosperms) and inedibility (mechanisms that hinder growth). The edibility of a food organism is indicated by the speed and efficiency of growth of the feeding organism. There is much evidence that the edibility of all living organisms has been lowered in many ways by the selective action of predatory and parasitic organisms, some of which are described in the following paragraphs.

A common selection effect is biochemical imbalance, either as a deficiency of a biochemical that is an essential nutrient for most heterotrophs or as an excess of some biochemical. For example, zein, the storage protein of *Zea mays* seed kernels, is extremely deficient in the "essential" amino acids, lysine and

tryptophan, but has a huge excess of glutamic acid. This makes corn an inadequate food for all except a few specially adapted insect pests.

The variety of imbalances that have resulted from random mutation and selection in plants is large and acts as a selective force on insects in two ways: (*a*) There is compensation for a specific imbalance by enhancement of specific biosynthetic or detoxication mechanisms, which will act inefficiently if the insect feeds on plants in which other types of imbalance exist; this kind of nutritional interlinking is a first step toward dietary specificity. (*b*) As imbalances become more prevalent and more extreme, there is selection for association between insects and microorganisms whose biosynthetic abilities can rectify the severe nutrient deficiencies of the plant. This symbiosis can be external and unspecific, as in the association of *Drosophila* with yeasts (38, 46) or of *Hylemya* with bacteria (54), but, as dietary restriction and specialization increase, symbiotes become internal and specific (144).

The devastating combination of the mechanical invasive power of the insect and the biochemical power of its symbiotic microorganisms is probably the selective force that has caused the proliferation of "secondary plant substances" such as the alkaloids, terpenoids, flavonoids, and so forth. Speculation about the function of these substances is curiously biased. Plant physiologists are baffled by their diversity and erratic distribution and think of them as metabolic errors or accidents. Plant pathologists are concerned with their possible role in resistance to fungi and other pathogens. Entomologists interested in chemoreception and host selection emphasize their potential attraction or repellence to insects. This review, having a nutritional bias, will emphasize their antibiotic action. It is very probable that most of the secondary plant substances serve one of these functions and not unlikely that some substances (being the result of many selection pressures) have several different functions.

All animals seem to have chemoreceptors that are involved in feeding reflexes, and both the receptors and the reflex arcs presumably undergo variation and selection, so that nutritionally adequate foods induce feeding and inadequate foods do not. This mechanism need not be—and probably cannot be—infallible. For example, the silkworm, *Bombyx mori* (Linnaeus), feeds on several plant species on which its growth and survival are very poor (53). Nevertheless, chemosensory feeding reflexes must predict nutritive value with a fair degree of accuracy. There is a positive correlation between food preference and the nutritive value of plants eaten by the grasshopper, *Melanoplus mexicanus* (Saussure) (139a). It is theoretically possible for a plant to develop a "pure" repellent, which has no physiological action other than inhibition of the feeding reflex in insects. However, it is more likely that repellents originate as genuinely noxious agents and that later they cause a selection for chemosensory mechanisms for their detection and avoidance. These mechanisms may also fail. For example, the nicotine in tobacco leaves acts as a repellent for *Leptinotarsa decemlineata* (Say) (53), even though this insect is highly resistant to the toxic action of nicotine (65). As in protective

mimicry, however, sensory deception and confusion must ultimately rely on the existence of real dangers, acting often enough (on the population, not the individual) to endow the instinctive reflexes with survival value.

If most secondary plant substances are selected for because they cause real damage to the metabolism of insects or their symbiotes, they must act as selective forces not only for avoidance mechanisms but also for mechanisms that can neutralize their effect. This must have happened in the tobacco hornworm, *Protoparce sexta* (Johanssen), for which nicotine is neither a repellent nor a toxicant (65). Some aphids that attack tobacco may avoid the repellent and toxic action of nicotine by selective phloem feeding. However, the imported cabbageworm, *Pieris rapae* (Linnaeus), is likewise neither repelled nor killed by nicotine, although it does not attack tobacco, but various crucifers. Cross-resistance to nicotine, both chemosensory and metabolic, can therefore result from selection by other agents.

Of the myriad of secondary plant substances, the nicotine alkaloids will be given emphasis in this review as a possible example of the biochemical interaction between insects and plants. It is true that nicotine is only a fragment of the complex unit that is the *Nicotiana* plant. The evolution of nicotine biogenesis must have involved many accidental steps and must be inextricably interwoven with the evolution of the many other substances, such as polyphenols (162), that play a vital role in *Nicotiana*. The economic value of tobacco, of which nicotine is a major, simple, active ingredient, has furthered the accumulation of more extensive data on the nicotine alkaloids than on other plant biochemicals.

## The Biogenesis of the Nicotine Alkaloids

Although *l*-nicotine is well-known as the major alkaloid of some species of *Nicotiana*, it occurs in other genera of the Solanaceae and has been isolated in small quantities from one or more genera of Leguminosae (101), Compositae (154), Asclepiadaceae (107), and even from pteridophytes (*Lycopodium*, *Equisetum*) (103). Alkaloids containing pyrrolidine or piperidine rings are widely distributed in the plant kingdom, and probably all arise from similar aberrations of the biosynthetic and degradative mechanisms for the amino acids ornithine and lysine (102, 150). In *Nicotiana*, the sidetracking of normal metabolism that gives rise to the nicotine alkaloids has been partly elucidated and is shown in Figure 1. The five basic reactions are: (*a*) synthesis of the piperidine ring from the lysine pathway (92, 94); (*b*) synthesis of an unknown pyridine-ring precursor from the nicotinic acid pathway (41); (*c*) synthesis of the pyrrolidine ring from the ornithine pathway (36, 43, 91, 93); (*d*) coupling of the pyridine ring with one of the unsaturated rings; and (*e*) reversible methylation of nornicotine to nicotine (28, 155). There are also degradative pathways for the alkaloid (95), and, while turnover is normally slow, it is accelerated by starvation or in excised roots, so that the alkaloids can be useful as an emergency reserve of nitrogen and carbon, (108).

Some of these pathways are under genetic control (40). All are present in

the roots of *Nicotiana glauca*, but (*a*) is deficient in the roots of most species of *Nicotiana*, which therefore contain very little anabasine. The shoots apparently lack all pathways except (*e*); nicotine synthetized in the roots is translocated to the leaves and demethylated to nornicotine, which is the dominant alkaloid in *Nicotiana sylvestris* and most other wild species (157). In the cultivated *N. tabacum*, demethylation does not normally occur in the leaves, and nicotine is the dominant alkaloid; however, when the leaves are air-dried, demethylation occurs in some varieties, which yield low nicotine (but high nornicotine) tobacco (182). These pathways are latent in the tomato, *Solanum lycopersicum*, since minute amounts of nicotine are normally present in

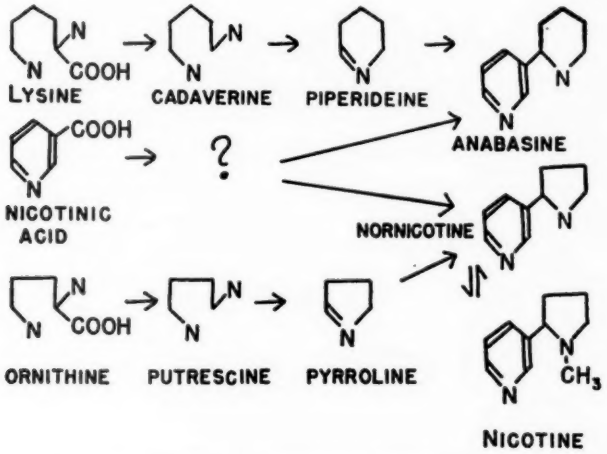


FIG. 1. Pathways from normal amino acid metabolism to the nicotine alkaloids in *Nicotiana tabacum*.

the leaves; unlike tobacco, tomato leaves lack storage mechanisms for accumulating nicotine, and tomato scions grafted on roots of *Nicotiana rustica* show toxic effects even though the nicotine content is lower than in tobacco leaves (183). On the other hand, tobacco scions grafted on tomato roots contain much more nicotine than is present in normal tomato leaves (173) and must in some way favor synthesis of nicotine (or precursors) by the roots. Seeds of the wild *Nicotiana glutinosa* contain nicotine, while those of some cultivated varieties of *N. tabacum* do not (172); synthesis begins in seedlings, and nicotine gradually accumulates during growth. The major alkaloid of the tomato, tomatidine (88), contains a piperidine ring, but this is condensed with a sterol nucleus instead of a pyridine ring (102). In other Solanaceae, the N-ring (by condensation with an unidentified metabolite) gives rise to the tropane series of alkaloids, such as atropine or hyoscine (scopolamine). Occa-

sionally, two distinct condensations occur in the same plant, which then contains two quite different alkaloids; for example, *Duboisia myoporoides* (Solanaceae) contains both anabasine and hyoscine, and *Anabasis aphylla* (Chenopodiaceae) contains both anabasine and lupinine (150), a quinolizidine ring alkaloid.

In some cultivated plants, selection by man has lowered the alkaloid content; for example, the edible pea, *Pisum sativum* contains only about 2 per cent of the sparteine and lupanine present in *Lupinus sp.* (187). Not all the mechanisms for alkaloid synthesis have been lost, however, since extracts of pea seedlings can form anabasine if cadaverine is added (125).

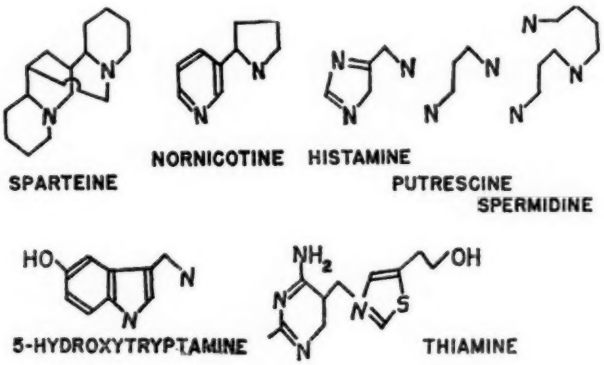


FIG. 2. Structural similarities of naturally occurring diamines, the action of which can be related to that of nicotine.

## THE PHYSIOLOGICAL ACTION OF NICOTINE ALKALOIDS

Nicotine belongs to the group of biologically active diamines (Figure 2) of which the simplest and most common is putrescine, the decarboxylation product of the amino acid ornithine. This is normally present in minute amounts in bacteria (170), fungi (63), insects (1), and mammals (151), usually as its more complex derivative, spermidine. The metabolic role is still obscure, but it is required at vitamin levels for growth of *Hemophilus parainfluenzae* (67) and a mutant strain of *Aspergillus nidulans* (160). Spermidine occurs in mammalian semen, where it may act as an antibiotic (143), since at high concentrations it is toxic to bacteria (85) and also to higher animals (169). The toxic secretion of the bird spider, *Pamphobeteus tetracanthus* Pocock, contains peptides of these diamines (50).

Another common active diamine is histamine, the decarboxylation product of histidine; the function of this substance in mammals is not perfectly clear, although it seems to be involved in complex injury and defensive reactions. It is one of the constituents of wasp venom (77).

Plants contain many diamines more complex than nicotine, such as sparteine, emetine, *d*-tubocurarine, and so forth. All the diamines have characteristic toxic actions on nerve cells. Although there is an abundance of literature describing these effects, especially in mammals, no clear picture of the fundamental action has emerged. The literature on neurotoxic action of nicotine alkaloids in insects has been well reviewed (112). They may act primarily on the surface structure of intact nerve or muscle cells.

Nicotine is not a typical diamine, since the pyridine nitrogen is only weakly basic (pK 6.0). It is therefore analogous to aromatic amines like 5-hydroxytryptamine, a neurohormone widely distributed in vertebrate and invertebrate animals, with powerful pharmacological activity, but not yet a clearly defined normal metabolic role; it also is a constituent of wasp venom (77).

Although nicotine has a variety of actions on nerve cells, in insects for which it is toxic the cause of death is paralysis of nerve centers not unlike that caused by the sting of hunting wasps. That it has other biological actions is certain, but these have seldom been observed because they are less dramatic, slower, and require higher concentrations. Some insects that are resistant to the quick-killing action of a single dose of nicotine may nevertheless be adversely affected by continuous intake of larger doses, by delayed growth, or by lowered fertility. This would be analogous to the action of piperonyl butoxide, which does not kill roaches and is efficiently detoxicated and excreted (153), but nevertheless strongly inhibits their growth (61). Nicotine at 0.01 to 0.05 per cent in the diet of rats does not cause obvious nerve poisoning or death, even though the daily intake would be quickly fatal if given as a single dose; it does cause severe growth inhibition of young rats (189). Nicotine impairs development of the chick embryo even at a very early state (86) and is toxic at 1:500 dilution to some bacteria (195). There is structural similarity between nicotine and the vitamin, thiamine, which can block some of the actions of nicotine in mammals (140). Other thiazole-ring compounds, such as sulfathiazole, are nicotine antagonists (192), and antagonism is shown by synthetic diethylaminoethyl esters, such as caramiphen (Parpanit®; β-diethylaminoethyl ester of 1-phenylcyclopentanecarboxylic acid) (69).

Some peculiarities in the toxic action of nicotine must be noted. The alkaloid in *Nicotiana* leaves is present as a salt with citric and other organic acids. The acute oral toxicity to some insects, such as *P. americana*, is only one-thirtieth of the toxicity of the free alkaloid acting by contact or as a fumigant (111). This may explain why some insects that attack tobacco, such as *Thrips tabaci* Lindeman or *M. persicae*, are susceptible to the fumigant action of nicotine (89, 145). The contact action of nicotine may be of no significance in natural selection but only a man-made phenomenon.

There are no definitive data on the true relative toxicity of the nicotine alkaloids to insects, since the few tests reported used sprays and the actual dosage entering the insects may have varied. The spray toxicity to *Aphis*

*rumicis* Linnaeus (66, 146) and *Aphis fabae* Scopoli (18) is in the order: anabasine > nornicotine > nicotine; anabasine is the least volatile and nicotine the most volatile, and this factor may be important, since extremely dilute (0.01 per cent) solutions were sprayed. When 1 per cent solutions were sprayed on the celery leaf tier (*Udea rubigalis* Guenée) or the large milkweed bug [*Oncopeltus fasciatus* (Dallas)], the order of toxicity was nicotine > anabasine > nornicotine (18), which is correlated with lipoid solubility (and probably penetration rate). When injected into *O. fasciatus*, nicotine and nornicotine are equitoxic (177). Tentatively, we may infer that there will not be large differences in the acute toxicity of the commonly occurring nicotine alkaloids; on the other hand, the fact that nornicotine predominates in the wild species of *Nicotiana* suggests that its chronic toxicity may be greater than that of nicotine.

## Natural Susceptibility and Tolerance to Nicotine and Other Insecticides

It would be illuminating to correlate the tolerance of many insect species to nicotine with tolerance to other insecticides and with the chemical composition of their natural diet. The available data (128) are too fragmentary to permit a truly critical correlation; nevertheless, it seems possible to classify insects into three groups. Group I is highly susceptible to nearly all contact insecticides and includes most aphids and the silkworm, *B. mori*. Group II is nicotine-tolerant but quite susceptible to many insecticides, such as rotenone, pyrethrins, and parathion. It includes many diverse species, such as *P. americana*, *Anasa tristis* (DeGeer), *O. fasciatus*, *Popillia japonica* Newman, *Tenebrio molitor* Linnaeus, *Apis mellifera* Linnaeus, and *Ceratomia catalpae* (Boisduval). Group III is relatively tolerant to all insecticides and includes mostly lepidopterans, such as *P. rapae*, *P. sexta*, and *Galleria mellonella* (Linnaeus); perhaps the new multiresistant strains of *M. domestica* fall into this group. There are no data on nicotine tolerance of the many strains of *M. domestica*, however.

Such a survey underlines the importance of three fundamental modes of resistance. Non-penetration into the insect, for example, makes *P. americana* and *A. tristis* immune to the contact action of rotenone, although they are highly susceptible by injection (111). If an insecticide passes through the cuticular barrier, it can be selectively destroyed and excreted before it can cause irreparable damage. Such detoxication presumably is involved in the specific nicotine-tolerance of *A. tristis* and is certainly a major factor in specific resistance of various trains of *M. domestica* to insecticides (24).

There are claims that resistance can be conferred by mechanisms other than detoxication, such as storage in the fat reserves of the body (72) or excretion of unaltered insecticide (138). It has not always been shown, however, that storage and excretion are not normal processes in the S strains or that equal doses of a non-toxic analogue of the insecticide are more efficiently stored or excreted by the R strain than by the S strain. Such a control is es-

sential because S insects are severely damaged by the insecticide, and any difference in metabolism between them and R insects can logically be attributed to the striking difference in their state of health (32). A control of this kind (132), using the non-toxic *alpha* isomer of BHC, provided conclusive evidence that a lindane-R strain of *M. domestica* absorbs the BHC isomers less than half as quickly and metabolizes them more than twice as quickly as a lindane-S strain. No excretion of unchanged lindane was observed. "Storage" (accumulation) of unchanged insecticide within the body was, of course, observed, but this is an inevitable result of the fact that the rate of penetration exceeds the rate of detoxication during the first few hours after application. Therefore, the fact that storage of *alpha*-BHC is greater in lindane-S than in lindane-R flies merely reflects the fact that the lindane-S flies absorb quickly and metabolize slowly.

A similar controlled experiment using both a toxic $S^{35}$ analogue of dieldrin and a non-toxic $Br^{82}$ analogue has shown that dieldrin-S and dieldrin-R *M. domestica* both store and excrete the unchanged compounds to the same extent and also metabolize them at seemingly equal rates (190). This suggests that some other, unknown mechanism is the cause of the resistance. Since there is cross-resistance between lindane and the cyclodiene insecticides, this unknown mechanism may also be present in lindane-R flies.

The insects in Group II seem to be specifically nicotine-tolerant (although most of them also are DDT-tolerant). There is no obvious correlation with diet, but it may be a cross-tolerance resulting from the relatively common occurrence of N-ring alkaloids. A specific detoxication mechanism for nicotine seems probable, and, although no data are available for insects (158), metabolism of nicotine occurs in bacteria, plants, and vertebrates (188).

The insects in Group III, showing generalized tolerance to insecticides, are unlikely to have exceptionally efficient detoxication mechanisms for all organic compounds. It seems more reasonable to invoke the somewhat misleading term "vigor" or, perhaps, the clearer term "endurance," which can be defined as the ability to survive for a long period with a severely stressed, malfunctioning or non-functioning nervous and muscular system and without food or water. The extraordinarily tolerant larva of the wax moth, *G. mellonella*, is not, as its normal dietary might indicate, a highly restricted feeder on honeycombs; it thrives on a mixture of dog meal (or yeast) and Pablum® (185) and would probably survive on a great variety of foods and and certainly with very little water. Mortality curves with pyrethrins (by injection) have an extraordinarily low slope, from 8 per cent kill at 0.1 μg./gm. up to 96 per cent kill at 1000 μg./gm., and show a plateau at about 50 per cent kill in the range from 30 to 300 μg./gm. (12). (A similar mortality curve for dieldrin on dieldrin-R mosquitoes (39) has been interpreted as evidence of a hybrid population consisting of genetically different subpopulations, one S and the other R.) It seems probable that *G. mellonella* and other Group III insects would be able to endure for long periods a deprivation of food and water that would kill many other insect species. It also is probable

that the adult insects, with more highly organized nervous systems, would be much less insecticide-tolerant. This difference between larval and imaginal stages is well-known in *M. domestica* and is also common with other insects, e.g., the fifth-instar larva of *U. rubigalis* is more than 20 times as resistant to nicotine as the adult (145). The "endurance" factor may also be important in lowering the susceptibility of larvae as they grow larger, e.g., the sixth-instar larvae of *P. eridania* are 10 times more resistant to nicotine than the second-instar larvae and 30 times more resistant than the unfed first-instar larvae (145).

Natural selection, therefore, seems to have endowed the larval forms of many insects, which must undergo variable and severe biochemical stress, with less vulnerable nervous systems and more active defense mechanisms than the adults, and this "endurance vigor" is of value in survival. Its apparent absence in aphids may be attributable to the sap-feeding mechanism, by which they avoid intake of many deleterious plant biochemicals. The absence of "endurance vigor" in *B. mori* is correlated with the extreme host specificity of this species (which is effectively an avoidance of exposure to most plant poisons) and perhaps also is a result of selection during domestication. Similar mechanisms may be latent in adults but are not actively selected in nature. The introduction of contact insecticides by man must have selected such mechanisms, but, since the nervous system of the adult cannot revert to the simpler larval level, protection is more readily achieved by selection of less permeable cuticle or more active detoxication enzymes.

Some selection for "vigor" is also probable; a relatively DDT-R strain of *P. americana* has been shown to withstand physical stress for a much longer period than a more DDT-S strain (68). Roaches can survive without food and water for two weeks or more, while less "vigorous" house flies usually die within two days.

The genetic and biochemical nature of the changes caused in populations of *M. domestica* by selection with organic phosphates and with DDT have been revealed by extensive and brilliant investigations, which are reviewed in the following sections. These changes are not easily interpretable or predictable from the nutritional factors that (this review assumes) have been influencing the S strains, because much less is known about the biochemical genetics of S strains than of R strains of *M. domestica*, and still less, about the normal nutritional factors. In fact, the biochemical ecology of insects is at present only in the stage of conception.

## Comments on Terminology Useful in Biochemical Genetics

There seems to be no inclusive term for the group of similar biochemicals, the synthesis of which is controlled by different alleles of the same gene. The term "allanes" is suggested here to include all the biochemical phenotypic variants determined by alleles of one gene. More specific terms could be coined by using the prefixes "alla" or "all" with root words, e.g., "allaproteins" could be "allenzymes" or "allantigens." Like the parent term, allele,

these terms would not be free of difficulties. For example, in microbial genetics, the mutant variant of an enzyme is often not considered to be an enzyme, since its turnover number may be so low that the usual tests detect no activity (49). There is also the complex phenomenon of apparent control of one enzyme by two non-allelic genes, as in the action of *rosy* and *maroonlike* on xanthine dehydrogenase in *Drosophila* (58). Nevertheless, the "allane" terms would avoid the diverse circumlocutions now used, e.g., "normal" and "abnormal" hemoglobins or an enzyme and its "isoantigenic" proteins lacking enzyme activity. In view of the striking similarities between certain groups of proteins controlled by non-allelic genes, perhaps a broader category of genes deserves to be recognized and called "polyalleles" or, briefly, "palleles." These would be identified not by a definite locus on a chromosome but by the similarity of the biochemicals ("pallanes," "pallaproteins," "pallenzymes," etc.) that they control. The hemoglobins and cytochromes are "pallanes" in the sense that their genes may have been alleles that ceased to be so because of chromosomal rearrangement. Even more impressive evidence of "pallanic" (or "pallelic") relationship is provided by the various esterases and peptidases that are inhibited by toxic organophosphate esters such as di-isopropyl fluorophosphate. The di-isopropylphosphoryl group forms a stable ester linkage with the amino acid serine; this linkage is located in the peptide chain sequence glycine-aspartic-serine-glycine in chymotrypsin, trypsin, thrombin, and phosphoglucomutase, but in the sequence glycine-glutamic-serine-alanine, in aliesterase and pseudocholinesterase (70, 78). The importance of "pallelism" is that it increases the number of mutant genes that can be maintained at moderately high frequency by removing them from direct competition with one allele of exceptionally high survival value.

## Detoxication of Organic Phosphate Esters by Insect Esterases

Resistance to organic phosphates in *M. domestica* is caused by an R gene (or closely linked genes) (129) that controls esterases that hydrolyze oxygen analogues (oxons) of many phosphate insecticides (7). Most "oxon-esterases" are variants of the "ali-esterase" present in S flies, and the R genes are alleles of the ali-esterase genes, (134, 135). Normal ali-esterase is very active on aliphatic carboxylic esters, such as methyl butyrate (turnover number[3] of the order of 100,000 per minute), and it has a high affinity for, and is strongly inhibited by, many oxons (5, 113). Enzymes of the oxon-esterase group are less active (turnover number for methyl butyrate about 1000 per minute) but are not strongly inhibited by most oxons. Instead, they can slowly hydrolyze some oxons (turnover number for diazoxon about 0.1 per minute)

[3] The turnover number of an enzyme is usually defined as the number of molecules of substrate acted on by one enzyme molecule in one minute, under optimal conditions of pH, temperature, substrate concentration, and so forth.

(7, 135). These oxon-esterases are not true phosphatases, but ali-esterases of low efficiency and broad substrate specificity. R strains selected by phosphate insecticides therefore usually are characterized by low ali-esterase activity (6, 15), coupled with an enhanced detoxication rate for certain organic phosphates.

There are several different oxon-esterases. In a malathion-R strain, resistance to malathion [and probably other "safe" phosphate insecticides (131), such as acethion] is very high, but there is little cross-resistance to other phosphates (27, 133). This oxon-esterase probably hydrolyzes the carboxylic ester linkage, not the phosphate ester. In R strains selected by Diazinon® [*O,O*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidyl) phosphorothioate], there is cross-resistance to aromatic phosphates, such as parathion, but only slight cross-resistance to aliphatic phosphates, such as malathion or dimefox (27). This variant oxon-esterase has a specific affinity for the aryl-phosphate linkage.

The specific cholinesterase of the insect nervous system is not altered in phosphate-R flies (7). Presumably, modification of its activity so impairs nerve function that the survival value of alleles of the cholinesterase gene is low. The normal function of ali-esterase is unknown but is less critical, since its oxon-esterase variants are viable. In fact, over 80 per cent of the ali-esterase can be specifically inhibited in S flies by tri (*o*-tolyl) phosphate without causing any obvious injury (163). Ali-esterase may be involved in metabolic reactions necessary for growth, such as synthesis and hydrolysis of cholesterol esters (30).

One Diazinon-R house fly strain has two R-genes, isolated by repeated backcrossing with an S strain (134). One is an allele of the ali-esterase gene and controls a "low ali-esterase" oxon-esterase that hydrolyzes both diazoxon and para-oxon. The other is not an ali-esterase allele, and it controls an enzyme that hydrolyzes only diazoxon (135); it may be an allele of the gene that controls the "aryl-esterase" present in S flies (5, 114). Aryl-esterase (or aromatic esterase) is an ill-defined unspecific enzyme that hydrolyzes aromatic esters, such as phenyl butyrate or para-oxon, and (unlike cholinesterase and ali-esterase) is not inhibited by oxons. It may be inhibited by piperonyl butoxide, which synergizes Co-Ral® [*O*-(3-chloro-4-methylumbelliferone) *O,O*-diethyl phosphorothioate] and its oxon (Coroxon®) in house flies (120).

## Role of Esterases in Detoxication of Pyrethrins and Carbamates

The pyrethrins are esters of complex cyclic alcohols and carboxylic acids. They are only moderately toxic to *M. domestica* ($LD_{50}$ range from about 25 mg./kg. for pyrethrin I to 100 mg./kg. for cinerin II), but the toxicity is augmented about tenfold by the addition of non-toxic synergists like piperonyl butoxide (74). Toxicity to many other insect species (*P. americana, O. fasciatus*) is higher (about 5 to 10 mg./kg.) (111), but only weakly synergized

by piperonyl butoxide (less than twofold) (59). Studies on *M. domestica* using $C^{14}$-pyrethrins proved that they are rapidly absorbed and detoxicated and that piperonyl cyclonene lowers absorption but inhibits detoxication (191). Since the primary metabolites have not yet been identified, it is not known whether the first step in detoxication is hydrolysis by an esterase. *In vitro* studies of pyrethrin detoxication so far have failed to solve this problem (20, 33); pyrethrins resemble DDT in being completely insoluble in water, and enzyme studies should make use of the efficient solubilization methods that were found to be necessary for the *in vitro* activity of DDT-dehydrochlorinase (97, 98, 99). The fact that each of the stereoisomers of allethrin is synergized by piperonyl butoxide two- to threefold, even though their toxicity varies 100-fold, indicates that the detoxication enzyme is indifferent to the *d*, *l*, *cis*, or *trans* configuration (56, 57). Synergism is maximal with esters of cinerolone, somewhat less with esters of pyrethrolone, and least with esters of allethrolone, suggesting that the point of attachment to (and perhaps of attack by) the enzyme is the allylic side-chain of the cyclopentenolone ring. The ease of hydrolysis of pyrethrins solubilized by lipoproteins (34) gives some support to the hypothesis that esterases detoxicate these insecticides. By selection of house flies with pyrethrins synergized with piperonyl butoxide, strains showing over twentyfold resistance are obtained (42), but no studies of detoxication in these R strains have yet been reported. A wild strain of *Blattella germanica* (Linnaeus) eightfold more resistant to pyrethrins and piperonyl butoxide has been found (83).

The carbamate insecticides are esters of phenols with N-methyl- or N,N-dimethyl-carbamic acid. They are structural analogues of acetylcholine, and many are strong and specific inhibitors of cholinesterase and also of the butyrylcholinesterase of mammalian blood plasma but are less active or inactive as inhibitors of ali-esterase or other esterases. Their toxicity to hemipteroid insects shows good correlation with *in vitro* anticholinesterase activity (87), but this is not true for roaches, house flies, or fruit flies (62, 148). The low toxicity of Sevin and compound Hercules-5727 (*m*-isopropylphenyl N-methylcarbamate) to house flies and roaches is greatly enhanced by synergists like piperonyl butoxide or sesamex (48, 61, 122). $C^{14}$-Sevin is rapidly metabolized in house flies and roaches to unidentified derivatives seemingly identical with those formed from $C^{14}$-1-naphthol, and this *in vivo* detoxication by a hypothetical "carbamate esterase" is blocked by sesamex (47). Although carbamate esterase is inhibited by piperonyl synergists, it is not the same enzyme that detoxicates pyrethrins, since carbamates (but not pyrethrins) are synergized by 2-(3,5-dichloro-2-biphenylyloxy) triethylamine (Lilly 18947) (124). This compound is not a specific carbamate synergist, however, since it blocks many different detoxication mechanisms in mammals (22), such as the procaine esterase found in human (but not in rat) blood plasma; procaine esterase is also strongly inhibited by carbamates like Pyrolan® [5-(3-methyl-1-phenylpyrazolyl) dimethylcarbamate] (142) and neostigmine [(*m*-hydroxyphenyl) trimethylammonium methyl sulfate di-

methylcarbamate] (130). The methylenedioxyphenyl derivatives are likewise unspecific and synergize many types of insecticides (16, 120, 149).

House flies and roaches must have a high level of several esterases (other than cholinesterase and ali-esterase) that can detoxicate a variety of lipoid-soluble esters; on the other hand, hemipteroids have a strikingly low internal level of esterases (113). The process of external digestion and subsequent ingestion of largely water-soluble substances that characterizes many suctorial insects will reduce the absorption of lipoid-soluble esters, and natural selection may then lower the internal esterase levels. This would explain the susceptibility to esters like pyrethrins and Sevin and also the fact that synergists do not enhance toxicity to Hemiptera such as the milkweed bug, *O. fasciatus* (62).

The high toxicity of heterocyclic carbamates such as Dimetilan® (3-methyl-5-pyrazolyl dimethyl carbamate) and Pyramat® (2-*n*-propyl-4-pyrimidyl dimethylcarbamate) to house flies and roaches indicates that the "carbamate esterase" that acts on Sevin cannot hydrolyze these carbamates, which differ from the benzenoid carbamates in being more polar and water-soluble, more resistant to alkaline hydrolysis, and much slower in combining with (and probably dissociating from) cholinesterase (31, 110). Combinations of Dimetilan or Isolan® [5-(1-isopropyl-3-methylpyrazolyl) dimethylcarbamate] with Sevin show slight synergism on house flies and roaches (62); this suggests that heterocyclic carbamates have some affinity for "carbamate esterase" and can partially inhibit it.

Normal S-strains of *M. domestica* contain a high percentage of individuals completely resistant to Hercules-5727 and to Sevin, and selection with these insecticides quickly gives rise to completely resistant populations (47, 55). DDT-R strains show cross-resistance to Sevin, and parathion-R strains show yet higher cross-resistance, extending even to very toxic carbamates such as Dimetilan (48). Malathion-R strains show very high cross-resistance both to Pyrolan and to Pyramat (90). Selection with Isolan, however, has given only a sevenfold increase in resistance (55). The carbamate-R strains show only minor cross-resistance to other insecticides, and methylenedioxyphenyl synergists partially counteract the resistance (48, 55). All these facts suggest that carbamate esterase activity may be found in allenzymes of the oxon- and aromatic esterase groups. In human plasma, Sevin and other N-alkylcarbamates are hydrolyzed by the albumin fraction, not by the butyrylcholinesterase or arylesterase of the globulin fraction, while dimethylcarbamates are not hydrolyzed (31).

## Detoxication of DDT and its Analogues

DDT was the first synthetic insecticide to be used on a large scale for house fly control and the first to give rise to highly resistant strains. It is fitting that, some ten years later, the detoxication enzyme for DDT in flies should be the first such enzyme to be isolated in highly purified form (97, 98, 99a). It is a small water-soluble protein (molecular weight about 36,000),

containing little or no lipid, nucleotide, or other bound coenzyme, but specifically activated by reduced glutathione or cysteinyl glycine, in the pH range 6.5 to 8.5 (optimum 7.4). The content in DDT-R flies is of the order of 1 μg. (30 μμM) per fly. The turnover number *in vitro* at 25°C. is only about three (97), but this represents a potential detoxication rate of 0.035 μg. of DDT per minute per fly, a rate much greater than that observed *in vivo*, since the rate of penetration of DDT topically applied to flies is only of the order of 0.002 μg. per minute.

When a similar isolation procedure is applied to the CSMA (Chemical Specialties Manufacturers Association) DDT-S strain of flies, the preparation shows DDT-dehydrochlorinase activity, but the activity is only 1/1000 of that in the DDT-R flies (97). The CSMA strain now has an $LD_{50}$ to DDT of about 0.3 μg./fly, a value significantly higher than that of S flies when DDT was first introduced (about 0.05 μg./fly). Nevertheless, it now seems certain that an enzyme that can dehydrochlorinate DDT has always been preadaptively present in many S strains of *M. domestica*, in *Epilachna varivestis* Mulsant, some mosquitoes, and even in man (24, 137). It is possible that the enzyme in DDT-R flies is identical with that in DDT-S flies but the amount present is increased 1000-fold, but it is more likely that (as in the case of ali- and oxon-esterases) the enzymes are slightly different and are controlled by different alleles of the same gene. The normal function of the S enzyme can vaguely be inferred from the fact that bis(*p*-chlorophenyl) trifluoromethyl carbinol is a growth-inhibitor for house flies (3), as well as a strong synergist for DDT in R flies (171) and that the actively growing larvae of S flies have higher DDT-dehydrochlorinase activity than the adults (121). This suggests that the S enzyme is involved in the metabolism of growth, possibly as a "dehydrase" acting on the *beta*-hydroxyacyl-coenzyme A derivatives of long-chain fatty acids to form $\Delta^2$-unsaturated-acyl-coenzyme A. It has been suggested that the DDT synergist, Dimite® (DMC; 4,4′-dichloro-*alpha*-methyl-benzhydrol) is dehydrated to its ethylene derivative (DME) (139), which may be the actual inhibitor of DDT-dehydrochlorinase, since this enzyme also rapidly converts 2,2-bis(*p*-chlorophenyl)-1-chloroethane to DME, which then inhibits the reaction (98). DME itself is an effective DDT synergist (106).

If this interpretation is correct, the DDT-dehydrochlorinase isolated from S flies has a very low turnover number (less than 0.01) for DDT, and DDT might act as an inhibitor of the reaction between this S-enzyme and its unknown normal substrate. This would not, however, be the long-sought enzyme (112), the inhibition of which is the primary toxic action of DDT, since non-toxic DDT analogues like Dimite would probably also inhibit it. The presence of a typical DDT-dehydrochlorinase in *E. varivestis* not selected by DDT (35) supports the hypothesis that the enzyme (or group of pallenzymes) has a metabolic function not identical with the dehydrochlorination of DDT.

The data on "DDT-dehydrochlorinase" in larvae and adults of S and

R strains of *M. domestica* (123), leave open the possibility that the R gene does not determine a qualitatively different enzyme, but only the quantity present. Its action would then be to cause a 1000-fold increase in the quantity of enzyme in the adult stage and presumably a smaller increase in the quantity present in the larval stage. Until the normal substrate of DDT-dehydrochlorinase is found, it will not be possible to determine whether the enzymatic activity on this substrate exactly parallels the activity in dehydrochlorinating DDT, thereby proving that there is no qualitative change.

The fact that a highly DDT-R strain of *M. domestica*, rich in DDT-dehydrochlorinase, can, by further selection with DDT plus synergists, give rise to a DDT-synergist-R strain with a much higher dehydrochlorinase activity (100a, 121) indicates that the quantity of enzyme may be controlled by more than one gene. Probably no gene acts in complete independence of all modifiers (23), and the assumption that resistant populations are simple mixtures of RR, RS, and SS genotypes is often likely to be incorrect.

It is now clear that many earlier reports of metabolism of DDT to 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) in various insect species are not valid (24), since other metabolites give colors similar to DDE in the Schechter-Haller reaction. The first non-DDE metabolite to be positively identified is 4,4′-dichloro-*alpha*-(trichloromethyl)-benzhydrol (already known as a synthetic acaricide, Kelthane®), which is formed from DDT in DDT-R strains of *Drosophila melanogaster* Meigen (174). Resistance in the Japanese strains is determined by a gene on the second chromosome (176). The detoxication enzyme must be an oxidase and the wild-type S enzyme may be involved in normal hydroxylation reactions (possibly of the sterol ring) necessary for growth. Since this pathway could act on chlorine-free DDT analogues like dianisyl-neopentane, it may be present in dianisyl-neopentane-R strains of *M. domestica* (25). Both DDT-S and DDT-R strains of *Drosophila* are resistant to dianisyl-neopentane (175). There is some evidence that the "DDT-oxidase" may not effectively detoxicate the nitro analogues of DDT, since a DDT-R strain of *B. germanica* shows cross-resistance to methoxychlor but not to Prolan® 1,1-bis(*p*-chlorophenyl)-2-nitropropane (37), although the primary metabolite of DDT in roaches has been tentatively identified as Kelthane (127, 175). All claims that DDE is formed in roaches are of doubtful validity; studies in our laboratory at Berkeley over many years indicate that, as methods of isolation and identification have improved, the presence of DDE has become less and less certain, and the most recent experiments on *B. germanica* show no trace of DDE (127).

It is curious that DDT pressure selects a dehydrochlorination enzyme in *Musca*, but an oxidation enzyme in *Drosophila*. This may be a mere coincidence, because of the small number of R-strains studied, and more extensive work may discover both types of detoxication in both fly genera. Their dietary is so different, however, that one might expect significant dif-

ferences in the nature of their "S enzymes" and in the frequency of various gene alleles, thus making selection of one mechanism much more probable. *Drosophila* is primarily a yeast-feeder (46), adapted to media rich in starch or sugars; the female lays eggs on fruit in which decay is only incipient and infects it with spores of the same yeasts that were its own larval food. *Musca* lays eggs on dung of any of a large number of vertebrates; the dung is already heavily infected with many bacteria and contains waste products from the great variety of plant and animal foods that the vertebrates have fed on. Such a heterogeneous environment must put a premium on genetic variability, at least in the genes controlling potential detoxication enzymes.

## The Significance of Growth Inhibitors

Work on insect-resistant varieties of cultivated plants (136) has shown that selection by man has often bred out of a species some factors that protect the wild strain from insect attack. A survey of over 3000 plant species (76) for possible toxic principles, however, shows that a large majority seems to be innocuous to many insects. This work is not definitive, since only aqueous extracts of many species were tested and many toxicants are water-insoluble. Nevertheless, we may tentatively conclude that quick-acting insecticides like nicotine and rotenone are not common defensive mechanisms in plants. Even when a quick-acting toxicant is present, it may have slower, less obvious effects, which are overlooked in the customary rapid screening process. Alkaloids of *Veratrum album* are growth inhibitors for first-instar larvae of *M. domestica* but have little effect on large third-instar larvae (13); this may be attributable to the relatively high food intake of the small larvae. The inhibition is less severe on a highly nutritious diet than on a poorer diet. The inhibition of oogenesis in adult *Musca* and *Habrobracon* by colchicine occurs at very low dosages where no other effects can be detected (64, 117). This is true also of the inhibition of oogenesis in *Musca* and in *A. aegypti* by the trifluoro analogue of Kelthane (3, 4) and probably also for related acaricidal DDT analogues. These acaricides may inhibit similar enzymes involved in growth of both insects and mites, but their effect on insects has been less noticeable because young, actively growing insects are seldom used in screening insecticides and, in any case, are not observed over a prolonged period of time (104). The slow toxicity of Kelthane to young nymphs of *B. germanica* has been noted following treatment of roach colonies with this acaricide to control infestations of mites (61). Sublethal doses of DDT shorten the life-span and lower the fertility of DDT-R *M. domestica* (73), and Ronnel® [*O,O*-dimethyl *O*-(2,4,5-trichlorophenyl) phosphorothioate] has a similar action on *Sitophilus oryza* (Linnaeus) (66a).

Equally interesting growth inhibition can be shown for compounds known to be synergists for pyrethrins or carbamates. Piperonyl butoxide (118) and many other 3,4-methylenedioxyphenyl derivatives (116) are powerful inhibitors of growth of *M. domestica* larvae. Piperonyl butoxide and "car-

bamate synergists" like Lilly 18947 [2-(3,5-dichloro-2-biphenylyloxy) triethylamine] and SKF 525-A (β-diethylaminoethyl diphenylpropylacetate) stop growth of young nymphs of *B. germanica* completely for many weeks without causing obvious toxic effects (61). These compounds block many oxidative, hydrolytic, and conjugation reactions involved in the detoxication of drugs by animals (22).

Podophyllotoxin, which occurs as a glucoside in the roots of several species of *Podophyllum* (166), is toxic to growing and dividing cells of plants and animals. In the rat, it is most toxic to embryos, less to young animals, and least to adults (44). The non-toxic alkaloid reserpine, from the roots of *Rauwolfia*, is also a growth inhibitor (29). An interesting group of isoflavones has been studied in various species of *Trifolium;* red clover, *T. pratense*, contains 7-hydroxy-4′-methoxyflavone (formononetin), which inhibits growth of the fungi *Sclerotinia trifoliorum* and *Fusarium nivale* (180), and also 5,7-dihydroxy-4′-hydroxyisoflavone (genistein), which has estrogenic action in mammals and can cause infertility in sheep (14). A related estrogenic compound, coumestrol, occurs in various clovers and in alfalfa (*Medicago sativa*).

The presence of relatively non-toxic substances like sesamin and sesamoline in the Rutaceae, or hibalactone and hinokinin in the Coniferae, is not explained by the odd fact that these methylenedioxyphenyl derivatives act as synergists for pyrethrins (109), which are not present in these plants. It now seems probable that these substances are growth-inhibitors. Their ability to synergize insecticides is an accidental consequence of the fact that these "growth enzymes" can slowly detoxicate insecticides. In some instances, the growth-inhibiting properties of substances occurring in plants have been discovered first in the search for factors conferring resistance to insect or microbial attack. Whole ground corn contains a growth inhibitor for *T. molitor* in addition to its deficiency of lysine and tryptophan (96). Benzoxazolinone was identified as an "anti-fusarium factor" in rye seedlings (179), and 6-methoxybenzoxazolinone, as an inhibitor for *Pyrausta nubilalis* (Hübner) in corn (10, 156); the latter also is a growth inhibitor for *B. germanica* and various other insects and fungi (11, 184). It is possible that some inhibitory actions on insect growth are indirect, the primary inhibition being on symbiotic microorganisms (164). In any case, the most common effect of selection by microbes and insects seems to be the formation of extremely broad-spectrum, weakly antibiotic substances that inhibit a variety of enzymes and slow the growth process. The inhibition may be so broad that the plant must protect itself against its own inhibitor, usually by forming an inactive derivative that will be activated by the digestive enzymes of its enemies. For example, the precursor of the benzoxazolidinones in the Gramineae is a glucoside (181). Many glucosides are hydrolyzed by digestive enzymes in the gut of the insect to liberate the active toxicant, but the plant often ensures the liberation by also providing the lytic enzyme, which becomes active when the cells are damaged. Thus, the amygdalin in

the bitter varieties of *Prunus amygdalus* is accompanied by the amygdalase and prunase necessary for its hydrolysis to hydrocyanic acid, and the scutellarin in the leaves of *Scutellaria baicalensis* is accompanied by the glucuronidase necessary for its hydrolysis to scutellarein (79).

### The Significance of Analogue-Synergism

Most of the secondary plant substances are accompanied by one or more structural analogues. All four known pyrethrin analogues show a similar toxic action, although the cinerins are less toxic (and less stable) than the pyrethrins. It can be predicted that a mixture of a pyrethrin with a cinerin will show some synergism, since the more stable analogue will tend to slow the detoxication of the less stable one. This "analogue-synergism" is a common phenomenon with synthetic insecticides on strains having detoxication mechanisms. DDT is synergized in DDT-R *M. domestica* by many of its structural analogues (167), including relatively toxic analogues like methoxychlor, which is only slowly metabolized by DDT-dehydrochlorinase. Bulan® [1,1-bis(*p*-chlorophenyl)-2-nitrobutane] is also a DDT-synergist (61) and, like the other synergists, forms a complex with DDT-dehydrochlorinase (80). Bulan apparently also synergizes its close analogue, Prolan, in Dilan-R *M. domestica*, since the $LD_{50}$ of Dilan (a mixture of one part Prolan and two parts Bulan) is 7 μg./fly, while that of Bulan is 11 μg./fly, and Prolan is >100 μg./fly (i.e., not attainable).

In an analogous study on lindane-R *M. domestica vicina*, it was shown that a partially purified BHC containing 60 per cent *gamma* isomer was more toxic than pure lindane (168).

Rotenone is more toxic to insects than its analogue, deguelin, and their 15-hydroxy analogues, sumatrol and toxicarol, have negligible contact toxicity, but all four analogues are highly toxic to goldfish (128). The toxicity of rotenone to *E. varivestis* is greatly enhanced by piperonyl butoxide (19), but the limited data make any claim that the rotenoid analogues might be mutually synergistic highly speculative. The data for the nicotine alkaloids are even less amenable to analysis, although nicotine can be synergized by a surprising variety of synthetic compounds (167). Although it might be expected that some substances in tobacco could (as a result of natural selection) work synergistically with nicotine, this has not been investigated. However, recent work on the cigarette beetle, *Lasioderma serricorne* (Fabricius), shows that growth on several kinds of dried tobacco leaves is depressed by high nicotine content, by deficiencies of essential nutrients, and by unidentified "material which enhances the toxicity of nicotine" (193). Plant poisons studied in isolation, like words taken out of context, may lose much of their effectiveness.

Although it is unlikely that synergism can restore the toxicity of synthetic insecticides to the very high level of the preresistance era, it is theoretically possible to hamper the selection process. For example, two toxic

structural analogues may be "mutally synergistic" if (like Bulan and DDT) they are detoxicated by different mechanisms but are similar enough so that each can block the mechanisms acting on the other (62). Although there are no data on the simultaneous selection of two different detoxication mechanisms, it may prove to be a problem in adaptation much more difficult for the insects to solve.

It also seems desirable to investigate the complex (and, if this review is correct, deleterious) dietary factors that are acting on insect species that are serious pests and to examine the possibility of aggravating the biochemical status by human action. This work would merge with that on insect-resistant varieties of crop plants and would consider the merits of alternation of plant varieties or even species. Programs of crop rotation are already useful in soil management and represent an additional tactic in insect control.

## Summary

Insects reared on any food that allows normal growth and development do not differ greatly in the degree of tolerance to contact insecticides. The cause of the two- to threefold difference that is often found cannot be elucidated with certainty. Continuous exposure, during growth, to sublethal levels of the insecticide may cause physiological adaptive changes that slightly increase tolerance.

Natural selection by insects (and other heterotrophic enemies) has enhanced various dietary imbalances and the synthesis of many "secondary substances" in plants, giving rise to many diverse malnutritious plants. Most insects are genetically adapted to survive on only a few kinds. The larval feeding stages of relatively polyphagous holometabolous insects often show extraordinarily high and generalized tolerance to contact insecticides; this is probably the result of selection for endurance of prolonged biochemical stresses of many kinds. The adult stages have more complex and vulnerable nervous systems and, usually, much lower insecticide tolerance, since they are less subject to biochemical stress.

Selection by contact insecticides increases the frequency of certain alleles of genes that control enzymes involved in the growth process. The R alleles are more efficient than the S alleles in causing detoxication of the insecticides and probably somewhat less efficient in normal growth metabolism. The detoxication enzymes that result from a relatively brief (10 to 50 generations) period of insecticide selection have extremely low turnover numbers, but this low efficiency is adequate to neutralize the minute quantities of insecticides that kill S insects and thereby to checkmate economical chemical control.

The origin of "detoxication enzymes" from "growth enzymes" may be correlated with the fact that most secondary plant substances act as growth inhibitors, not fast-acting nerve poisons. Natural selection has therefore favored enzyme structures that are unspecific enough to destroy many of the

diverse structures occurring in the natural growth inhibitors, and variation in the food plants probably preserves a variety of alleles of varying specificity within the insect population. Selection of these, probably with modifiers that augment their viability, accounts for the surprisingly rapid (on the evolutionary time scale) adaptation to contact insecticides. Since these alleles function well in normal growth, their selection has an aspect of unspecificity, cumulativeness, and permanence that may hinder, and will certainly complicate, the future progress of chemical control.

## Addendum

It has been shown by F. Saba of the University of Bonn (personal communication) that adaptation of the two-spotted spider mite, *Tetranychus urticae* Koch, to five widely disparate host plants caused the substrains to differ only slightly in resistance to tetraethylpyrophosphate (TEPP). Selection with tetraethylpyrophosphate for 12 generations gave rise to low-resistance strains ($LD_{50}$ increased five- to tenfold) on *Phaseolus vulgaris*, on *Dianthus caryophyllus*, and on *Prunus domestica* as host plants, while on *Lamium album* and on *Humulus lupulus* the mites became highly TEPP-resistant ($LD_{50}$ increased 50- to 200-fold). Following 11 generations without TEPP treatment, TEPP-resistance remained very high on *Humulus lupulus* but declined to a fivefold level on *Lamium album* and almost completely disappeared on *Prunus domestica*. This work provides impressive evidence that diet strongly affects the adaptive value and equilibrium frequency of relatively rare alleles that, under insecticide selection pressure, give rise to resistant strains.

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# PRINCIPLES OF INSECT COLD-HARDINESS[1]

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## INTRODUCTION

Cold is a relative term which for present purposes will be considered to encompass temperatures too low to support normal development of the insects concerned. The effects of cold on insects may be manifold, but a primary distinction must be made on the basis of whether freezing does or does not result. The dividing line, however, is seldom at the freezing point of the tissues but rather at the supercooling point, for insects almost invariably supercool to some extent, usually many degrees.

Cooling without freezing has been termed "chilling" (9), particularly when the emphasis is on temperatures above 0°C. Such investigations usually deal with insects that do not normally encounter cold, e.g., household pests. Unaccustomed to cold, they tolerate it poorly. On the other hand, insects that hibernate in temperate and colder climates must tolerate a wide range of low temperatures, often for long periods. Some species improve their tolerance through processes of cold-acclimation and cold-hardening.

All insects supercool to some extent, regardless of need; in fact, some that need to do so least supercool below −40°C. Most hibernating insects do need to supercool, however, to avoid freezing, which would be fatal; the remainder have not this need because freezing is not injurious to them. The former group are called freezing-susceptible and the latter freezing-resistant or freezing-tolerant (34).

The principles of insect cold-hardiness will be discussed under three main headings: (*a*) chilling and cold-acclimation, (*b*) avoidance of freezing by supercooling, and (*c*) freezing-tolerance.

## CHILLING AND COLD-ACCLIMATION

Insects hibernating in cold regions are generally able to withstand fairly low temperatures for long periods of time. Under natural conditions, the only mortality directly attributable to temperature is from freezing, whereas those insects that survive are free of any chilling injury. Nevertheless, if the chilling is artificially prolonged, it becomes harmful and eventually fatal.

Most insects hibernate at a particular stage in their life cycle, one that is usually more cold-hardy than the preceding stage, so that a comparison indicates that cold-hardening has taken place. This is literally true, but the use of the term "cold-hardening" in this sense spoils it for description of additional hardening during hibernation. It has been suggested by Salt (34)

[1] The survey of the literature pertaining to this review was concluded in January, 1960.

that the term be restricted to improvements in cold-hardiness during hibernation; the same argument applies to cold-acclimation, which in one sense is synonymous with cold-hardening.

As the temperature falls, the needs of the insect change from those of activity, growth, reproduction, etc., to those of survival. Temperature and time are here inseparable, for, in general, cold injury becomes more severe as the temperature is lowered or as the time of exposure is increased. The injury is thought to arise when some segments of the metabolism get out of balance with others, the differential effects thus producing an unbalanced, unhealthy organism. This is frequently characterized, when in sublethal doses, by such things as loss of co-ordinated movement, failure to molt, or the appearance of morphological abnormalities after ecdysis.

Acclimation is a word that is used with many shades of meaning, some of which are not suitable for our present purpose. Much of the work with poikilotherms has dealt with the rates of various reactions (metabolism, activity, response to stimuli) before and after acclimation. This is the viewpoint of a review of temperature compensation in poikilotherms by Bullock (10). As an example of this type of adjustment, the cockroach *Blattella germanica* (Linnaeus) becomes more active at a given low temperature after acclimation at a somewhat higher low temperature than without such acclimation (14). Stated in other terms, after cold-acclimation the insects could remain active at lower temperatures than otherwise. Throughout the present review, however, we shall be chiefly concerned with survival and mortality at low temperatures, and so we shall regard cold-acclimation from the standpoint of its effect on cold injury rather than on activity.

Very few studies have been made of the acclimation of insects to low temperatures from the standpoint of survival. It is not known whether many insects show such a response, and whether it is related to the needs of the species. Atwal (8) demonstrated it in pupae of *Anagasta kühniella* (Zeller), which cold-acclimated rather rapidly. The criterion was survival after a nonfreezing exposure to −15°C. for four hours. Acclimation was maximal in about four hours, but deteriorated after about 16 to 48 hours. It increased as the temperature (constant in his experiments) was lowered to 10°C., but was less at 5°C. At the latter temperature, injurious effects of chilling were considered to have begun to offset the advantages of acclimation. Atwal found that the pupae were most cold-tolerant and acclimated best when newly formed, with a progressive decline thereafter.

The rapidity of thermal acclimation is such that some response is almost immediate. In a natural situation with temperatures fluctuating above and below an activity threshold, the insect is continually adjusting physiologically (acclimating), and, of course, this is reversible. In fact, cold-acclimation may be considered as the loss of heat-acclimation, and vice versa.

A paradoxical situation appears to exist while an insect is being acclimated during mild chilling: the cold eventually becomes injurious, yet the

acclimation is beneficial and probably offsets the injury to some extent. However, the benefits of acclimation depend on what is to come, or, in other words, acclimation is a potential advantage that may or may not be used. It is only utilized if the insect is exposed to a lower temperature, and it is measured in terms of advantage over a less acclimated individual or over its own former self. Chilling injury, in contrast, accumulates in a positive manner, but, as long as it is slight, acclimation remains of potential value to the insect.

## AVOIDANCE OF FREEZING BY SUPERCOOLING

Supercooling is not necessarily dangerous to an insect, for the risk of freezing varies from virtually none to virtual certainty in response to several factors, particularly temperature and duration. The fact of being supercooled is in itself harmless, but, if freezing follows, it is an entirely different matter. Some insects can survive freezing, but most cannot; for the latter, the supercooling point represents the lethal limit of low temperature. However, this is not a fixed temperature, for it is influenced by several factors and is also subject to the laws of probability. Nevertheless, a few empirical tests will permit fairly accurate prediction of results under a variety of conditions.

### Nucleation and Freezing

It is apparent that our real concern is not with supercooling as such but with the freezing that suddenly terminates it. Freezing is initiated by an ice-crystal nucleus, which is formed in response to certain conditions. The following discussion of the application of nucleation theory to the freezing of supercooled insects is taken almost entirely from a paper by Salt (37).

As the temperature is lowered below the freezing point, the molecular arrangement of water becomes more and more icelike. The molecules slow down, they come closer together, and the probability of a chance orientation into the crystal structure becomes greater. It is thought that aggregations of molecules are first formed and that these grow or shrink in response to conditions, particularly temperature. Freezing does not begin until an aggregation attains a certain critical size, when it becomes a crystal nucleus. Ice formation is then assured by the growth, by accretion, of the crystal nucleus. In insect tissues the rate of this growth is usually fast enough to advance the ice front throughout the body in a fraction of a second. The probability of additional nuclei forming in this short time is slight, and, in any case, of little import. However, crystal growth is slowed down in proportion to increasing viscosity, so that in a rather viscous system many nuclei may form. In extreme cases, as in very dry systems, growth may be so slow that the crystals never reach a visible size; if the conditions that formed the first nucleus persist, the result is innumerable ice crystals that are a little larger than nuclei, invisible to the eye and optical microscope, but detectable by x-ray diffraction. The same effect can be produced by cooling very small

amounts very rapidly to extremely low temperatures. Innumerable nuclei are produced, but they are denied time to grow, which they do better at higher temperatures. The viscosity increases also as the temperature is lowered. The result is vitrification rather than freezing in the usual sense of the word.

Supercooling may persist for long periods if conditions are not sufficiently favorable for nucleation. Further cooling, however, will increase the probability of nucleation and eventually make it virtually certain.

This description of nucleation has assumed that only water molecules are used in building a nucleus. When this is so, nucleation is said to be homogeneous. Freezing in insects, however, probably involves heterogeneous nucleation, in which the nucleus is formed by the addition of supercooled water molecules onto a foreign (i.e., non-aqueous) surface. The more closely this surface resembles the structure of ice, the more efficient it is as a nucleating agent. Water can seldom, if ever, be completely free of such surfaces, whether they are the surfaces of suspended particles or the walls of a container, and, of course, water in a biological tissue is intimately associated with a wide variety of particulate matter. Since the nucleating efficiencies of surfaces vary, the limit of supercooling is actually imposed by the most efficient surface present. Because nucleation also depends on the probability of a particular molecular orientation, it follows that the number of "most efficient" nucleating agents present and the size of the water mass, as well as time, influence nucleation. Furthermore, a nucleating agent will nucleate at different temperatures, depending on whether it is inside a mass of water or in its surface layer, and on the influence of surface forces on it.

In basic studies, all these factors have to be carefully considered, but, in practical entomological studies, the situation is somewhat simplified, as little or no control can be exercised over the indigenous nucleating agents, surfaces and interfaces, shape, and size. A standard procedure for cooling is adopted and care is taken that only insects of similar stage, condition, background, and so forth are included in a single experimental group. The most efficient nucleating agents will determine the supercooling points for these conditions. Sufficient repetition will indicate the statistical variability and a mean supercooling point, which are valid for the specific experimental conditions. Thus, one can compare species, stages, treatments, background, and so forth without much concern about nucleating agents. However, to interpret results intelligently and to predict them in advance requires an appreciation of many aspects of nucleation theory. Such application during the past few years has placed the subject of insect-freezing on a much more substantial basis than formerly. Three examples will be considered in detail: (*a*) nucleation in the digestive tract by food and its contaminants; (*b*) nucleation in contact water of various types and the probability of inoculation through the cuticle; and (*c*) the influence of time on the probability of nucleation.

*Nucleation in food in the digestive tract.*—Some of the most efficient nu-

cleating agents known are mineral particles blown about in the atmosphere in the form of dust. Any object exposed to the air is bound to acquire dust, and, if it happens to be eaten by a chewing type of insect, the dust goes along too. Once inside the insect's digestive tract, the dust particles come into contact with water and become potential sources of freezing. As their nucleating efficiency surpasses that of the indigenous nucleating agents within the tissues, they rob the insect of much of its supercooling potential or cold-hardiness. This explains the general observation that feeding insects are less cold-hardy than their non-feeding stages. Exceptions to this rule may be explained by feeding habits; e.g., if the food is never exposed to the air, no contamination results. Internal parasites fall into this latter category; e.g., mandibulate *Scelio* larvae feeding inside grasshopper eggs. Piercing and sucking insects give variable results, probably dependent on the cleanliness or air-tightness of their feeding. Feeding mites, thrips, and some leaf miners gave low supercooling points almost consistently, whereas other species of leaf miners, aphids, mirid bugs, mosquitoes, and others gave variable results (40). However, the type of feeding and contamination of the food is not the whole story, for the food itself is freezable and may possess indigenous agents of high efficiency. In addition, maceration or other subdivision of the food produces aqueous masses which, by virtue of their surface characteristics in particular, raise the efficiency of nucleating agents within them above the level possessed in the intact food.

The nucleating efficiency of food and of its contaminants is soon reduced by passage through the digestive tract. Chemical changes are undoubtedly responsible in part, but it is also thought that the surfaces responsible for nucleating soon becomes coated with proteinaceous and other substances that "smother" them (37).

Before an insect molts it stops feeding and the fore- and hind-guts are emptied in readiness for ecdysis, which includes their ectodermal linings. The mid-gut is not so affected and can therefore retain its contents. Before and during a molt, the supercooling points of larvae of *Agrotis orthogonia* Morrison and *Anagasta kühniella* (Zeller) fall but rise again to the feeding level as soon as feeding recommences (33). It appears that much of the food's original nucleating efficiency is lost before it reaches the mid-gut. However, when feeding larvae are starved, supercooling is not increased as might be expected. In *A. orthogonia*, the gut contents were retained with little or no passage during starvation, but they became more watery at the expense of the tissues (33). Possibly the smothering action is not effective under such wet conditions, or perhaps the redistribution of body fluids creates situations involving fairly flat water surfaces. Considerable evidence indicates that small bodies of water with flat surfaces will not supercool as much as equivalent bodies with curved surfaces. It seems that forces operative in the surface layer strongly influence nucleation. It could scarcely be otherwise in a phenomenon so dependent on molecular orientation and arrangement. In

biological tissues surface layers are legion; here, the important characteristic is curvature, for any irregular aqueous surface is a mosaic of curved surfaces. The nature of the other component of the interface, i.e., whether gas, solid, or liquid, may also be important, though this is unknown.

*Nucleation in contact moisture.*—This is somewhat analogous to the situation just discussed, where food was often shown to reduce cold-hardiness. Aqueous material in contact with the exterior of the insect will do the same if the ice formed in it penetrates or grows through the cuticle, thus "inoculating" the inner tissues. At first glance this does not seem a likely event, for the cuticle of most terrestrial insects is rather waterproof. Nevertheless, the waterproofing is somewhat short of perfect, and any discontinuity is a possible site of inoculation, provided a continuous bridge of water penetrates the cuticle. Experimentally, inoculation through the cuticle is often an erratic response that seems to indicate a large element of chance in respect to location, but no precise work on this aspect is known. It has been observed, however, that inoculation takes place more readily at low subfreezing temperatures than at higher ones. Why this is so is not known.

Moisture on the exterior of a hibernating insect may come from various sources: condensation and contact with a moist environment are common in nature, whereas in the laboratory secretion, excretion, and wounding may occur as a result of handling.

The remarks already made concerning contamination by airborne particles and the influence of water surfaces on their action apply also to contact moisture. Both food and contact moisture, then, can reduce cold-hardiness seriously. However, few insects feed during hibernation, so that only those caught by an early frost would be endangered; furthermore, the hibernation quarters of a species are generally satisfactory for its needs, even though many individual sites may fall short through greater exposure to cold or moisture.

*Influence of time of nucleation.*—Because nucleation depends on the probability of a particular molecular event, the time required for it to actually occur is variable. It is insufficient, for example, to say that an insect freezes at −20°C.; it either remains at −20° for some time before freezing or else it freezes as soon as its temperature falls to −20°C. The first situation involves variable time at a particular temperature, whereas the second involves variable temperature (falling) at a particular time ("as soon as" or zero time). Because most experimental studies have utilized falling temperatures, the influence of time received little or no recognition until its importance was demonstrated by Salt in 1950 (32).

At a fairly rapid rate of cooling, an insect may freeze at, say, −20°C. If the rate is reduced, then clearly the time spent at temperatures approaching −20°C. is lengthened, and the insect will probably freeze at a slightly higher temperature because of this. In practice, no influence is detectable at rates of the order of a few degrees per minute, or even minutes per degree, but,

when cooling is extended over hours, days, or longer, the effects in terms of higher supercooling points are considerable. Maintaining such slow rates of cooling is difficult and unnecessary; instead, the material to be tested may be placed at a series of constant temperatures and the time taken to freeze each specimen recorded. Under natural conditions, of course, neither temperature nor rate of cooling is constant; as they fluctuate, so does the probability of nucleation.

Many hibernating insects spend long periods supercooled, and their chances of freezing in any particular period of time are of practical interest. The probability increases, of course, as the temperature is lowered and as time is lengthened. Continuing the example above, a rapid drop in temperature results in freezing when the insect reaches −20°C.; but, if the temperature falls short of this minimum by some amount, the insect may still freeze in time. At −19° it might take a minute to freeze, at −17° an hour, at −15° a day, at −10° a month. The time to freeze thus represents a measure of the probability of nucleation at a particular temperature. However, the element of chance ensures considerable variability in the actual results.

Since the relation between probability of nucleation and time is not linear, mean time values are not suitable for comparing the probabilities of freezing at different temperatures or in different species or populations. Instead, the median time value, i.e., the time required to freeze half of a given uniform population, is used.

Under ideal conditions, the relation between time and probability of nucleation is essentially that expressed by the compound interest law. A convenient form for our purpose is:

$$y_t = y_o e^{-rt} \qquad 1.$$

where $y_t$ is the number of specimens unfrozen at time $t$ out of an original population $y_o$, $e$ is the base of natural logarithms, and $r$ is the probability expressed as rate of freezing. If we convert Equation 1 to logarithms, we get:

$$\log y_t = \log y_o - rt \log e. \qquad 2.$$

As this equation is of the first degree in terms of log $y_t$ and $t$, a plot of these two terms must be a straight line. It is important to remember that $y_t$ is the number unfrozen and not the number frozen.

Figure 1 shows the relations between freezing and time for hibernating larvae of *Cephus cinctus* Norton exposed to −20° and to −24.5°C. The failure of the experimental data to be linear does not mean that the probability of freezing is not expressed by the formula. There is a simple reason for its divergence, which always follows the same trend in any population that is not perfectly homogeneous. Naturally, the insects with the most efficient nucleating agents, and therefore the greatest probability of freezing, tend to freeze first. Their freezing leaves the population poorer in terms of freezing probability, thus reducing the freezing rate, $r$. The result is a continual lower-

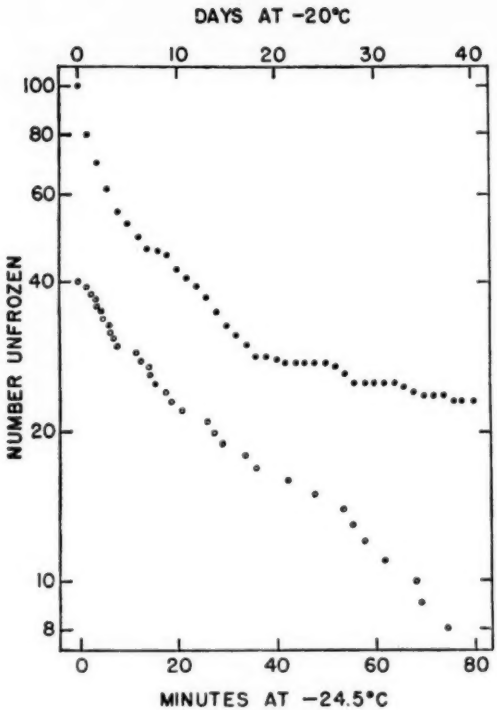


FIG. 1. Time-to-freeze curves of hibernating larvae of *C. cinctus* exposed to constant temperatures of −20°C. (upper curve, 100 larvae) and −24.5°C. (lower curve, 40 larvae).

ing of freezing rate as time goes on. Chance, of course, makes this an uneven decrease in an actual situation, but the over-all trend to lower rates is an inevitable consequence of heterogeneity of material.

### LIMIT OF SUPERCOOLING IN WATER

There is a large body of literature dealing with attempts to purify and supercool water as much as possible (26). Collectively, this work indicates a sort of limit near −40°C., attainable only with very small amounts of extremely pure water cooled fairly fast. To achieve supercooling points around −40°C., contamination was very carefully reduced, and very small droplets or capillary masses ( $<10\mu$ diameter) of water were used; this ensured that no droplet or capillary mass contained many foreign nucleating agents. The highly curved surfaces of the small masses of water tended to further sup-

press nucleation within their force-field, perhaps throughout the entire mass at such dimensions. (Even homogeneous nucleation would be suppressed by the restriction of molecular motion at the surface.) With future refinements in technique, it may be possible to supercool water more than this. However, because the probability of nucleation increases very rapidly as the temperature is lowered, in order to supercool water much below −40°C. we would have to cool infinitesimal amounts at incredibly fast rates. Hence, −40° seems likely to remain a near-limit of supercooling for pure water. In mixed aqueous systems, on the other hand, it is possible to increase supercooling by inhibiting nucleation. This occurs when molecular travel is restricted by hydrogen bonding, as in viscous syrups or gels. Though it may seem that high viscosity and gel structure act as mechanical barriers, both are directly caused by hydrogen bonding. Looking for viscosity effects, Lusena (21) failed to find evidence of increased supercooling in glycerol solutions up to 2.5 *M;* however, he limited supercooling to 1.02–2.36 C.° by means of silver iodide. In contrast, Salt (38) reports that supercooling of *Bracon cephi* (Gahan) larvae ranged from 26 to 36 C.°, and the amount varied in direct proportion to the solute concentration, most of which was glycerol. Though Lusena was of the opinion that viscosity did not affect supercooling in his solutions, Salt felt that the viscosity of the entire biological system would be great enough at the low temperatures involved (−35° to −45°C.) to be responsible. This is largely because the glycerol has a dual effect; i.e., in lowering the freezing point, the glycerol also increases supercooling at least an equivalent amount, but, since the viscosity increases with falling temperature, nucleation is inhibited somewhat and a further amount of supercooling is to be expected.

Insects that supercool to temperatures below −40°C. have all been found to have low freezing points attributable to high solute concentrations. Thus, a larva of *B. cephi*, which supercools to −45°C., will have a freezing point of about −15°, so that the amount of supercooling is only about 30 C.° (38). These are freezing-tolerant insects. In contrast, freezing-susceptible insects have freezing points only a degree or two below 0°C.

### Influence of Dehydration on Cold-Hardiness

In freezing-susceptible insects, increasing cold-hardiness is synonymous with falling supercooling points. Water loss or dehydration does lower supercooling points to the extent that it concentrates solutes and thereby lowers freezing points. Supercooling points fall an equivalent amount, plus a small additional amount because of increased viscosity. That there is some cold-hardening is indisputable, but the amount is usually negligible. This point was elaborated by Salt (34), who showed that water loss must be extreme before supercooling points fall appreciably. If this happens without harming the insect, then cold-hardening has certainly occurred; however, most insects cannot tolerate the high electrolyte concentrations resulting from such exten-

sive water loss. No cases are known where insects prepare for hibernation by dehydrating to the extent that their freezing points are lowered by several degrees.

It is nevertheless commonly assumed that dehydration appreciably increases the cold-hardiness of tissues and whole organisms, and, indeed, good correlations between them are frequently obtained. However, the relationship is only valid to the extent covered in the preceding paragraph, and the correlations are chiefly attributable to combinations of the following sets of circumstances: (*a*) Cold-hardiness is usually greater in hibernating than in other stages because, as explained more fully elsewhere in this paper, the non-feeding hibernator is more cold-hardy than its former feeding self; the glycerolated hibernator is more cold-hardy than its former non-glycerolated self. (*b*) Water content is usually less in hibernating than in other stages because of gut-evacuation, cessation or decrease of various secretory activities, accumulation of non-aqueous storage materials in the fat body, and consequent reduction of need for water in other tissues.

Nor is there any sound evidence that dehydration allows better survival after freezing. On the contrary, larvae of *C. cinctus* that had lost 20 to 30 per cent of their original weight by drying at 5°C. survived freezing much more poorly than did controls kept at 5°C. without drying. They were frozen, by inoculation, for one hour at −10°C. (40). Less ice was formed in the dry larvae, yet they suffered much greater injury than the controls. The osmotic concentration of the unfrozen body fluids would of course be the same in both groups at −10°C.

## FREEZING-TOLERANCE

The ability of some insects to survive ice formation inside their bodies has been known for over 200 years, since the days of Réaumur. Accounts of lepidopterous larvae, in particular, surviving after being frozen to the extent of brittleness are common in the literature. All of these species hibernate in relatively exposed situations, usually above the snow level, where they must be able to survive freezing or else supercool to below the minimum temperatures of their environment. Supercooling in insects is limited to about 30–35 C.°, so that, if air temperatures much lower than −30°C. (−22°F.) are to be tolerated, an insect must have a low freezing point in addition to maximum supercooling ability or else be able to tolerate freezing. So far as is known, the only low freezing points found in insects are the result of high glycerol concentrations, which also confer freezing-tolerance.

### Theories of Freezing-Tolerance and Freezing Injury

*The bound water theory.*—Before the discovery of glycerol in the haemolymph of *Hyalophora cecropia* (Linnaeus) by Wyatt & Kalf in 1956 (43), no satisfactory theory existed for the tolerance of freezing that is observed in

some insects. A serious attempt in this direction was made by Robinson (29, 30, 31), who invoked the concept of bound water, which was currently receiving much attention. Robinson was able to show a correlation between bound water and cold-hardiness in three species chosen to represent three levels of cold-hardiness: the cold-hardy *Antheraea polyphemus* (Cramer), the moderately cold-hardy *Callosamia promethea* (Drury), and the non-cold-hardy *Sitophilus granarius* (Linnaeus). This work was a correlation at best, and Robinson's whole case was based on it. The distinction between cold-hardy and moderately cold-hardy was vague and was unsupported by survival or mortality data. Furthermore, Robinson assumed that the free water in his insects was completely frozen at −20°C., when, in fact, his own data show that many of the insects were not frozen at all, but supercooled, at this temperature (30).

There was little agreement among workers in different fields on what the term "bound" water included, on where it began and free water ended, and, consequently, on the conditions under which it could be measured by satisfactorily standard comparative methods. The several methods in use lacked agreement when tested on the same material.

Later, Ditman, Voght & Smith (15) determined the percentages of water frozen in seven species of insects whose cold-hardiness varied greatly. Using the calorimetric, or heat of fusion, method, as did Robinson, they showed conclusively that the percentages of water remaining unfrozen at various temperatures bore no relation to the cold-hardiness of the insects. About 5 to 15 per cent of the water remained unfrozen in all species at temperatures below −30°C.; although some of this would freeze at still lower temperatures, the remainder might be considered "bound."

The theory of the protective action of bound water was explained by Robinson (29). Bound water was considered to be oriented as layers of hydrogen and hydroxyl ions absorbed alternately on the colloid micelles, and hence compressible, whereas free water is practically incompressible. The disruption of the cells by the expansion of water on freezing was regarded as the cause of freezing injury. Robinson (29) states:

> When adsorbed water undergoes compression, the colloidal particles and the bound water together occupy less space than the two separately. Therefore when free water freezes, as it does even in some hardy species, there will be more space for expansion without causing injury.

This explanation could only hold if water-binding occurred simultaneously with freezing, whereas it was considered to precede winter freezing as an autumnal cold-hardening process. The space gained by the contraction of the bound water would have to endure as empty or gas-filled space in order to be available for the future expansion of free water on freezing!

*The electrolyte-concentration theory.*—This theory assumes that if the

electrolyte concentration of a cell or tissue is increased, it eventually becomes lethal. The further assumption that this is synonymous with freezing injury is supported by some evidence, but also refuted by some. The theory has the advantage of easy comprehension, for it is obvious that, as the amount of ice increases in response to falling temperature, the remaining, unfrozen solution becomes more concentrated. At the same time, there are associated phenomena that could themselves be sources of injury, though some of these may be direct results of electrolyte concentration, e.g., the precipitation of proteins. In the same sense, dehydration could be considered synonymous, i.e., the removal of water as solvent is equivalent to concentrating the solute.

The salt-concentration theory has also the advantage of being quantitative, so that results should be predictable, within reasonable limits, for a given set of conditions. It has appeared to explain adequately the freezing injury of frog muscle (28) and the survival of human red blood cells (18, 19, 20). The theory gained much popularity when Lovelock used it to explain the action of neutral solutes, such as glycerol, in protecting red blood cells from freezing injury (20). More will be said about this in a later section dealing with the protective action of glycerol.

Levitt (17) has vigorously attacked the theory and claims that Lovelock's own data disprove it. To some extent this depends on interpretation, but it is true that some of Lovelock's results are unexpected. For example, the hemolysis of red blood cells in hypertonic NaCl solutions is much less than that produced by freezing in dilute NaCl to temperatures at which the unfrozen solution has equivalent hypertonicities. However, if the cells are resuspended in dilute NaCl after the hypertonic treatment, the hemolysis is similar to that produced by equivalent freezing. Discrepancies may possibly be explained on the basis of changing permeabilities of cell membranes to various ions, but nothing concrete in this respect has yet been demonstrated. The arguments for and against the theory are too extensive to be covered here, and the reader is referred to the original papers.

*The mechanical theory.*—It seems quite likely that the stresses and strains imposed by ice formation can harm biological tissues. The theory is quantitative to the extent that injury may be expected to be proportional to the amount of freezing or to occur only after a tolerance limit has been exceeded. However, it derives its support from qualitative observations and logic. Mechanical damage is expected because it seems a natural consequence of freezing, and, when one observes the freezing of biological tissues under a microscope, the mechanical aspects appear very impressive. This is especially true of plant cells, which generally show the disruptive effects of freezing much more distinctly than the more flexible cells of animal tissues (1, 27). A disadvantage of the theory is its lack of specificity. Cells vary in many ways and so do the mechanics of ice formation, especially when both are viewed on a micro-scale. This makes it very difficult to define injurious combinations and circumstances.

The modifications of ice formation produced by glycerol are such as to appear favorable, i.e., less harmful. Freezing is slowed down, and the crystal protrusions are more blunt and rounded. Less ice is formed at a given subzero temperature, of course, but, if a comparison were to be made at equivalent ice-volume levels, only shape and location of crystals would likely be significant. Reducing the speed of ice formation may produce less injury, but how this may come about is conjectural.

Single cells, or minute multicellular organisms in suspension in a medium may be considered to escape the mechanical action of ice by being pushed ahead of the ice fronts, or by being enfolded by them, thus remaining in the unfrozen portion. In such circumstances, the dimensions of the cells or organisms in relation to the dimensions of the interstitial unfrozen solution would be the determining factor. Since this hypothesis seems inadequate for organized animal tissues, it may serve as a warning not to base a general theory of freezing injury on data obtained solely from small dispersed units such as bacteria, blood and sperm cells, etc.

*The site-of-freezing theory.*—According to this theory, intracellular freezing is invariably fatal, whereas extracellular freezing is innocuous up to a point. The nature of the injury, when it occurs, must be explained by some other theory; hence, the site of freezing is only part of the story. The theory has enjoyed wide support from botanists, and, most recently, from a group of Japanese workers, particularly Asahina (1, 2, 3, 5, 6, 7), who turned from plant to insect and other animal tissues.

Freezing usually begins in the intercellular spaces in plants, and, if it penetrates the cell walls to freeze the cell contents, it has always been observed to be lethal (1, 17). If it does not penetrate, water leaves the cells in response to the increased osmotic pressure of the unfrozen portion of the intercellular water. The cell thus shrinks from within and is crowded from without, as well as being dehydrated. It can stand a certain amount of this but succumbs if a limit is exceeded. With falling temperature, the electrolyte concentration, the protoplasmic dehydration, and the spatial strains caused by the ice itself all increase, but which, if any, causes the injury is not known. As salt concentration and dehydration will be the same wherever the ice is formed, they do not appear to be the primary causes.

In animal cells, the situation is sufficiently different, particularly with respect to cell wall rigidity, vacuolization, and intercellular space, that the above description of extracellular freezing in plants is not necessarily suitable for animal cells and certainly not for insect cells that have been supercooled appreciably. Freezing of biological tissues occurs under a wide variety of conditions that influence its nature, particularly on a micro-scale. The freezing that takes place after 20 or 30 C.° of supercooling certainly differs in many ways and produces different results from that which occurs slightly below the freezing point. It is very difficult to initiate freezing in an insect at a temperature close to its freezing point, but, when it is possible to

curtail ice formation to a very small amount, the damage is comparatively slight. Under ordinary conditions, however, supercooling amounts to at least a few degrees, and three-fourths or more of the body water will be rapidly converted to ice. The velocity of crystallization and the structure of the ice formed will be influenced by temperature, solutes, colloidal matter, viscosity, thermal conductivity, and so forth. [For details, the reader is referred to excellent accounts by Luyet and his co-workers (22 to 25), which impress one with the importance of the spatial or mechanical aspects of freezing, even though one may favor a theory in which the ultimate injury is chemical in nature.]

When an insect of suitable translucency supercools appreciably and then freezes, a wave of whitening can be seen to sweep throughout the body from the point of origin, the ice-crystal nucleus. Although this first wave takes only a fraction of a second, additional freezing follows at an exponentially decreasing rate. Luyet (22) aptly calls the initial freezing "invasion" and the subsequent freezing "occupation." The invasion phase quickly permeates the entire aqueous mass with a feathery skeletal ice structure; this contributes heat of crystallization which, except for that portion lost to the environment, raises the internal temperature toward the freezing point. Meanwhile, the removal of solvent water as ice lowers the freezing point. Thus, a constantly changing complex results from the addition of ice and the formation of heat of crystallization, the subtraction of heat by the surroundings, and the concentrating of the unfrozen portion which depresses the freezing point; all of which slow down as the temperature differential decreases. Since these events follow the creation of only one ice crystal nucleus, a single, though highly ramified, ice mass is produced that could justifiably be called a single ice crystal. Regardless of the name, however, it is clear that when an insect freezes after supercooling, say, 20 C.°, the single nucleus grows to an ice mass of well over half the total body water in a small fraction of a second and approaches 80 to 90 per cent at an exponentially decreasing rate. The probability of more than one nucleus forming is at first remote, and, in any case, unless additional ones form during the invasion phase, they could scarcely change the crystallization picture.

It seems unlikely that such a picture, which applies to freezing-tolerant as well as to freezing-susceptible insects, could be the result of extracellular freezing alone. Cell walls, membranes, and protoplasmic structures of various kinds apparently do not stop or turn aside the advancing ice front. Until recently, there was no direct evidence that this was so, but recent observations on *Eurosta solidaginis* (Fitch) larvae have demonstrated that the fat cells freeze internally (39). These larvae supercool only about 8–10 C.°; insects supercooling more than this would be even more apt to freeze intracellularly.

The same article (39) also presents the first positive refutation, in insects,

of the theory that intracellular freezing is always fatal (39). It was demonstrated that the fat-body cells of larvae of *Eurosta* freeze internally when the insect is frozen after a normal amount of supercooling, and that it is water, and not lipid, that is solidified. As the overwintering larvae normally freeze and thaw many times without harm, intracellular freezing of the fat cells is a normal event of their hibernation. The cells, of course, survive. If, in view of the foregoing, the site-of-freezing theory is restricted to non-glycerolated (or equivalent) tissues, it perhaps may be as applicable to animal tissues as it appears to be to plant tissues. Since glycerol or sorbitol, or both, have thus far been found in all freezing-tolerant insects when looked for, it seems likely that such substances are responsible for their survival. All other insects are killed by freezing, and, since they probably freeze intracellularly when supercooling has been at least several centigrade degrees, intracellular freezing may indeed be the cause of injury. The action of glycerol may be to confer tolerance to intracellular freezing; since we are not yet certain how glycerol protects, this is a distinct possibility, supported by the evidence in *Eurosta* larvae (39) and by the probability that intracellular freezing usually occurs after appreciable supercooling.

Asahina has been a strong proponent of the site-of-freezing theory and has presented very convincing photograph. and other evidence in its favor, not only in plant cells, but also in insect tissues (1, 5). With the exception noted above—perhaps more accurately described as a special category than as an exception—the site-of-injury theory has much to commend it. However, it is unfortunate that Asahina has weakened his case by occasionally using the theory in reverse, claiming that intracellular freezing must have occurred when cells were killed or extracellular freezing when they survived (2, 3, 5, 6, 7).

As already mentioned, the site-of-injury theory is incomplete in that the ultimate cause of injury is not explained. It is much more compatible with the mechanical theory of freezing injury than the electrolyte concentration theory.

## Glycerol and Cold-Hardiness in Insects

In 1956, Wyatt & Kalf reported that they had identified free glycerol in the haemolymph of diapausing pupae of *H. cecropia* (43). Chino (11) found that in eggs of *Bombyx mori* (Linnaeus) glycogen was converted to glycerol and sorbitol upon entry into diapause and resynthesized when diapause was ended. Salt (35) found glycerol in hibernating *E. solidaginis* larvae and *A. polyphemus* pupae, which are freezing-tolerant, and also in *Loxostege sticticalis* (Linnaeus) larvae, which are not. Of 17 stages of 10 species of insects that are killed by freezing, *L. sticticalis* larvae alone possessed glycerol. *E. solidaginis* was found to contain sorbitol also (40). Later, Salt reported very high concentrations of glycerol, more than 5 *M*, in overwintering larvae of *B.*

*cephi* and showed its dual effect on the cold-hardiness of this species (36, 38). Similar concentrations of glycerol have since been found in other hibernating freezing-tolerant insects (40).

The discovery of glycerol in insects naturally promised a better understanding of their freezing-tolerance, since glycerol had, in the preceding decade, come into wide use for preserving spermatozoa, blood cells, etc., in the frozen state. This has been partially realized, for reports of the findings of glycerol in additional species are becoming commonplace, and it now appears likely that all freezing-tolerant insects, and perhaps other invertebrates as well, contain glycerol, or some equivalent, as a protective substance. The question of concentration will be discussed later, but first the theories of the mode of action of glycerol should be mentioned.

Lovelock (20) tested the ability of a number of neutral solutes, including glycerol, to protect human red blood cells from hemolysis after freezing. In previous work, he had concluded that freezing-injury is caused by high electrolyte concentration in the unfrozen portions of the system (18, 19). He points out that the addition of neutral solutes, by lowering the freezing point, reduces the electrolyte concentration of the unfrozen residue at any given sub-freezing temperature. Hence, the cell can be cooled more than formerly before the harmful concentration is reached. Levitt's criticism (17) of the electrolyte-concentration theory has already been discussed, and it is apparent that much more needs to be known about this complex subject.

Supporters of the theory that freezing injury is mechanical can point to the effects of glycerol and similar substances on the type and velocity of ice formation. Glycerol, for example, slows the rate of ice formation and modifies the structure of the ice; however, many substances will do this without conferring freezing protection. The evidence that such effects are protective is as yet only conjectural and is not amenable to precise experimentation. A possibility that occurs to the reviewer is that glycerol owes its protective action, in part at least, to its viscosity. Cell plasm containing glycerol in addition to its hydrophilic colloids and other substances may be fairly viscous near its supercooling point; after freezing begins, the unfrozen fraction becomes more concentrated and its viscosity may become very great. The result is that, upon freezing, the ice-front advance, particularly the occupation phase, is greatly retarded. This allows time for further nucleation which, in a very viscous fluid, tends to result in large numbers of nuclei growing very slowly (40). The further concentration that is produced augments the viscosity effect. This picture offers an explanation of Asahina's latest work (3, 41), wherein he obtained survival of two species of insects, a nematode, and glycerolated oyster tissue at liquid oxygen temperatures after prefreezing at temperatures near $-30°C$. A preliminary requirement was that the organisms could survive the prefreezing treatment itself, which they apparently did as a result of glycerol. [Both insects, *Monema* (*Cnidocampa*) *flavescens* Walker and *Papilio machaon hippocrates* Felder, contain glycerol (41) and

the oyster tissue was glycerolated. The nematode, *Aphelenchoides ritzemabosi* Schwartz, probably contains it also.] The action may be pictured as follows. The initial non-injurious freezing at −30°C. produces a viscous unfrozen residue. The sudden cooling of this residue to extremely low temperatures produces multinucleation with little or no crystal growth. The formation of innumerable nuclei in this fashion, to the extent that the water is all converted into nuclei or units very little larger than nuclei, produces the condition known as vitrification (23). This type of "freezing" is generally harmless to biological tissues.

Although the way in which glycerol protects is uncertain, there is no doubt that it does so under some circumstances. As yet we do not know whether freezing-tolerance is proportional to glycerol concentration in insects. With blood and sperm cells dispersed in a glycerolated medium, the relation holds approximately, up to a point where the cells cease to tolerate the glycerol itself (20).

Glycerol has been found whenever it has been looked for in freezing-tolerant insects but in only one freezing-susceptible species (Table I). With

TABLE I

OCCURRENCE OF GLYCEROL IN INSECTS

| Species and stage | Approximate maximum concentration | Reference |
|---|---|---|
| A. Freezing-tolerant | | |
| *Hyalophora cecropia*, pupal blood | 0.3 molar | 44 |
| *Hyalophora cecropia*, pupae | 1% of fresh wt. | 40 |
| *Antheraea polyphemus*, pupal blood | 0.05 molar | 44 |
| *Antheraea polyphemus*, pupae | 1.3% of fresh wt. | 35 |
| *Bombyx mori*, eggs | 1.1% of fresh wt.* | 11 |
| *Eurosta solidaginis*, larvae | 4.3% of fresh wt.† | 35 |
| *Pyrausta nubilalis*, larvae | 1% of fresh wt. | 40 |
| *Bracon cephi*, larvae | 25% of fresh wt. | 36, 38 |
| *Eurytoma gigantea*, larvae | 25% of fresh wt. | 40 |
| *Mordellistena* sp., larvae | 25% of fresh wt. | 40 |
| *Monema flavescens*, larvae | 2% of fresh wt. | 41 |
| *Papilio machaon hippocrates*, pupae | 0.5% of fresh wt. | 41 |
| *Camponotus pennsylvanicus*, adults, eggs | 10% of fresh wt. | 16 |
| *Pyrausta nubilalis*, larvae | — | 16 |
| *Melandrya striata*, larvae | — | 16 |
| B. Freezing-susceptible | | |
| *Loxostege sticticalis*, larvae | 4.3% of fresh wt. | 35 |

* Plus 2.2% sorbitol.
† Plus 4 to 5% sorbitol.

the exception of this one species, *L. sticticalis*, there is a qualitative relation between freezing-tolerance and the presence of glycerol, but to establish a quantitative relation much more data are required. Freezing-tolerance must be expressed in terms of survival of specific temperature-time exposures and these related to the concentrations of glycerol or similar substances. The information in Table I is clearly inadequate for this purpose, for no quantitative or comparative basis of freezing-tolerance is contained in it, except that one may be fairly certain that the three species that contain up to 25 per cent glycerol are very freezing-tolerant. Even here, the relation is complicated by the fact that the glycerol lowers the supercooling point to such a large extent that the insect is extremely cold-hardy from that standpoint alone. It may be that the smaller concentrations are adequate for protection against freezing injury and that the extremely high concentrations are not wholly necessary.

### Relation of Glycerol to Diapause

Only hibernating insects have so far been found to possess glycerol. They synthesize it in time for winter but not necessarily as soon as they enter hibernation. *B. cephi* larvae begin to make glycerol four to six weeks after cocoon-formation under natural conditions (38). Most species appear to lose glycerol rapidly as soon as post-hibernation or post-diapause development begins (5, 16, 38). Chino (12) stated that the resynthesis of glycogen from glycerol and sorbitol in *B. mori* eggs is:

> directly associated with the "break" of diapause *per se* and not with the subsequent process of post-diapause development, because when once diapause breaks, eggs can regain their glycogen content even when embryogenesis is suppressed by a temperature of 5°C.

However, the relation to diapause appears to be coincidence arising from concurrent timing, for, as far as is known, glycerol is not involved in the hormonal control of diapause. It has commonly been assumed that diapause is causally related to cold-hardiness in general, but here also concurrent timing is the only evidence. The diapause condition may have attributes, such as inactivity and lack of feeding, that favor winter survival, but the two factors that really determine cold-hardiness are supercooling potential, in most insects, and presence of glycerol, etc., in the others, neither of which is directly attributable to diapause. Asahina (4) related diapause to frost-resistance in the slug caterpillar, *Monema* (*Cnidocampa*) *flavescens*, and concluded that diapause contributes to cold-hardiness. However, the paper was written before he was aware that the freezing-tolerance of the species was attributable to glycerol, the presence of which explains his results better than invoking the vague attributes of diapause.

About three-fourths of a recent book by Ushatinskaya, *Principles of Cold*

*Resistance in Insects* [(42) in Russian[2]], is devoted to diapause, with such a diffused view of insect cold-hardiness as to be unrealistic in many respects. However, it thoroughly covers the practical side, as typified by records of winter mortality and survival.

[2] I am grateful to J. A. Shemanchuk of the Research Station, Lethbridge, Alberta, and W. Hanec of the University of Manitoba, Winnipeg, Manitoba, for translations of pertinent sections of this book.

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# THE BIOCHEMISTRY OF INSECT HEMOLYMPH[1]

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The hemolymph, or blood, of insects possesses properties that have attracted to it a major share of the attention of insect physiologists. Research on this fluid falls historically into roughly three phases. Investigations between 1864 [Landois (175)] and about 1915 established the general properties of hemolymph, with particular concern for pigments, the darkening phenomenon, and possible roles of the blood in respiration. This early work is reviewed by Schulz (246). From about 1925 on, quantitative methods, chiefly borrowed from clinical blood analysis, were applied more or less systematically to insect hemolymph, thus building up a body of data from which some significant patterns could be discerned. It became evident that insect hemolymph differs profoundly from human blood and that analytical methods developed for the latter are often inadequate to cope with its complexities. The findings of this period have been reviewed by Maluf (198) (whose article, however, contains serious errors), Timon-David (275), Rapp (233), Wigglesworth (288), and, most fully and recently, by Buck (42) and Chauvin (53). In addition, the earlier quantitative work on the silkworm was tabulated by Yamafuji (303), and a new comprehensive compilation of data on insect hemolymph will appear shortly (3).

Two earlier Japanese papers, which have escaped previous reviews except that of Yamafuji (303), deserve special mention. Tsuji (282), in 1909, using large volumes of silkworm blood, discovered several features not generally recognized until many years later. He demonstrated the role of oxygen in the darkening phenomenon, the presence of amylase and catalase, the abundant magnesium and the preponderance of potassium over sodium, and the presence of high levels of several organic acids, and he isolated crystalline tyrosine and leucine. Bito (27), in 1927, contributed the first systematic quantitative analyses on silkworm blood and showed changes in organic and inorganic fractions accompanying development and starvation.

In the last few years, new methods of microanalysis, especially those involving chromatography, have revealed a number of previously unsuspected components (sometimes at high concentration) of insect hemolymph and

[1] The survey of the literature pertaining to this review was concluded in April, 1960.

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have given more accurate quantitative knowledge. The present article will emphasize developments since Buck's review (42), and will also consider some of the earlier work, as well as some problems still unresolved. Since insect hemocytes have recently been reviewed by Wigglesworth (290), we shall be concerned solely with the plasma. In most of the chemical work, cell-free plasma has been used. The proportion of cells in insect hemolymph is so small, however, that they would contribute little to the composition of the whole; thus, results obtained with the whole fluid may be included without special comment. Some recent papers by Sissakian and co-workers on components and metabolism of "coelomic fluid" from silkworm pupae are not discussed, since this fluid, obtained by crushing whole pupae, contains products from the cellular tissues (254, 255, 257).

In order to emphasize the differences from the blood of vertebrates and to avoid misleading connotations, it may be found preferable to designate the body fluid of insects as "hemolymph" rather than "blood."

## General and Physical Properties

*The volume of hemolymph in insects.*—In order to estimate the contribution of the hemolymph to the reserves or metabolism of an insect, it is essential to know its volume, which varies greatly in different species and stages. In Lepidoptera, the abundant blood of the pupa may be utilized until almost none remains in the emergent adult (111, 129). Hemolymph volume is discussed by Beard (20) and Chauvin (53). An improved method for measuring it is by dilution of $C^{14}$-inulin, which has also been applied to determine, by difference, the amount of intracellular water (37, 185).

*Dissolved matter.*—The extent to which solute concentration varies in hemolymph is reflected in the wide range of specific gravity and osmotic pressure (42, 53). An extreme example is the Japanese caterpillar, *Monema flavescens* Walker, in which the water content of the hemolymph falls from 90 per cent in the feeding larva to 70 to 75 per cent in the overwintering prepupa; at the same time, the freezing point falls from −0.75° C. to −2.04° C., returning in the spring to its initial value (8). These changes may reflect the production and re-utilization of some solute such as glycerol (see below).

Two recent works have contributed to knowledge of osmotic regulation in insects. Zwicky (306) has shown that larval *Drosophila melanogaster* Meigen can regulate the osmotic pressure, chloride, and glucose of its hemolymph when placed in hypo- and hypertonic media. Marcuzzi (200, 201) has demonstrated that changes in ambient relative humidity may influence the freezing point of *Tenebrio molitor* Linnaeus hemolymph (range: −0.8° to −1.3° C.) and the concentrations of several components. Another indicator of total dissolved matter, recently measured for *Bombyx mori* (Linnaeus), is refractive index (154, 161, 270).

*Hydrogen-ion concentration.*—The numerous determinations of pH compiled by Buck (see 28; 42) have recently been supplemented by measurements on the hemolymph (as well as the gut juice) of 25 species, chiefly

Canadian forest insects, by Heimpel (126, 127). Mean values for all but three of these fell in the range 6.4 to 6.8. For the larch sawfly, *Pristiphora erichsonii* (Hartig), he observed a transient rise in pH during molting, thus confirming an effect earlier noted by Demjanowski *et al.* (73) during a very thorough study of pH in *B. mori*.

The buffer capacity of *Bombyx* hemolymph has been measured by Reali (235) and found to exhibit a U-shaped curve with pH similar to that previously demonstrated for *Prodenia eridania* (Cramer) (15) and *Gasterophilus intestinalis* (DeGeer) (183); buffering is minimal near the physiological range, in reverse of the pattern in mammalian plasma. The buffering is the sum of many contributing systems; with present knowledge of hemolymph composition, we can add to previous suggestions about these systems. In the physiological range, in addition to bicarbonate and inorganic phosphate, organic phosphates (pK 5.8 to 6.5) and histidine (imidazole pK 6.1; concentration in *Bombyx* up to 25 m*M*) are undoubtedly important. On the acid side, carboxyl groups of amino acids and other organic acids, especially citric, would be the major buffers, while, on the alkaline side, the amino groups of amino acids and other substances (e.g., phosphoethanolamine) would cause buffering. Protein, of course, also acts as a buffer over a wide range.

*The role of hemolymph in gas exchange.*—Apart from certain chironomids that possess a hemoglobin, the role of hemolymph in gas transport is minor: the oxygen carried is limited to that in physical solution, and the carbon dioxide consists of free dissolved $CO_2$ together with the carbonic acid-bicarbonate equilibrium system (182). Direct tracheation of the respiring cells provides efficient gas exchange. Recent work on the discontinuous respiration, or cyclic $CO_2$ release, of diapausing lepidopterous pupae, however, has given new significance to the hemolymph as a site of $CO_2$ storage. The explanations of this phenomenon by Buck (44) and by Levy & Schneiderman (190) depend upon the fact that the body water has far greater retention capacity for $CO_2$ than for oxygen. In *Agapema galbina* (Clemens) pupae, nearly half of the body water is in the hemolymph (46), which, when freshly drawn, contains four to 10 volumes per cent of $CO_2$ (45). Rapid interconversion of $CO_2$ and $H_2CO_3$ is catalyzed by carbonic anhydrase, which, although generally absent from insect hemolymph (109, 187), is present in the cellular tissues (5, 45, 260).

## Inorganic Components

*Inorganic cations.*—The inorganic constituents of insect hemolymph are of special interest because of their variable proportions, which often differ from those found in other animal groups and conflict with some generalizations that have been made about the uniformity of the extracellular medium of animals (17, 67).

Analyses prior to 1952 have been compiled by Buck (42) and by Duchateau, Florkin, & Leclerq (97). The latter paper also presents original deter-

minations of some or all of the elements Na, K, Ca, and Mg in the hemolymph of 63 species of insects. Additional analyses have been reported on *Periplaneta americana* (Linnaeus) (10), *Chortophaga viridifasciata* (DeGeer) (18), the aquatic larva of *Sialis lutaria* (Linnaeus) (248), *Oncopeltus fasciatus* (Dallas) (207), *T. molitor* (49), and eight other insect species (232). In addition, Ca and Mg have been measured in the hemolymph of two roaches (9) and 23 species of insects (66), and the literature on calcium and magnesium in insects has been reviewed in detail by Clark (63).

For purposes of comparison, the proportions of the four major cations may be expressed as indices that are percentages of their total in ion equivalents. Among the species listed by Duchateau *et al.* (97), such indices exhibit the following ranges: Na, 0.6 to 87.8; K, 1.6 to 53.4; Ca, 2.6 to 37.6; Mg, 3.4 to 73.5; while the values for total base range almost tenfold, from 42 to 413 m.eq./l. In interpretation of this diversity, Boné (29) first pointed out a relation to diet: zoophagous insects tend to have high sodium and phytophagous species high potassium in their hemolymph. This was supported by Tobias (276) who also showed that *Periplaneta* is capable of a substantial degree of regulation to maintain its normal Na:K ratio. The matter has been discussed at length by Duchateau *et al.* (97), who present a hypothesis involving both phylogenetic and dietetic considerations. The primitive pattern in insect hemolymph, they believe, is a high sodium type not dissimilar from that of other animal groups and exemplified today by the Odonata and, with some modification (often increased magnesium), many Orthoptera and Diptera and certain Coleoptera. The only apterygote insect examined to date, the thysanuran *Petrobius maritimus* Leach, conforms with this generalization, having the unusually high Na:K ratio of 40 (192). In the most advanced groups—certain Coleoptera (Chrysomelidae) and the Hymenoptera and Lepidoptera—which have evolved along with the angiosperms, we find progressive adaptation to a plant diet, the degree of regulation required of the insect being presumably reduced by maintenance of high potassium and magnesium levels in its hemolymph. Certain Lepidoptera and Hymenoptera that have secondarily abandoned phytophagy nevertheless retain the cation pattern of their vegetarian relatives. Curiously enough, almost all insects concentrate magnesium from their food (97).

*Ion binding.*—It has frequently been suggested that the metallic elements in insect hemolymph exist, in part, not as free ions, but as various organic complexes (18, 26, 42, 66, 143, 181). The evidence for this is chiefly indirect: (*a*) Attempts to prepare physiological solutions with inorganic components in the concentrations found in insect hemolymph led to precipitation of calcium and magnesium phosphates (26); when, however, amino acids were included at the biological concentrations, this difficulty disappeared (302). (b) Injection of salts of magnesium into locusts in amounts approximating those already present in the blood produced paralysis (180). (*c*) When Ringer solutions composed of chlorides of the cations in various proportions

were tested for maintenance of isolated insect hearts, the presence of magnesium was toxic, even though the blood of the species used is rich in this element (18). (*d*) The specific conductivity of hemolymph of *Pieris brassicae* (Linnaeus) is considerably lower than would be expected from inorganic analysis (32, 33). (*e*) Hemolymph contains substances known to be effective in forming complexes with divalent metals; these include proteins, amino acids (especially histidine), di- and tricarboxylic acids (especially citric), and phosphates (inorganic and organic).

Despite the apparent importance of these phenomena, direct investigation of ion binding of insect hemolymph seems to be virtually restricted to the recent paper of Carrington & Tenney (52). Using hemolymph of *Antheraea* (= *Telea*) *polyphemus* (Cramer), these authors could find no evidence for any binding of potassium, while some 15 to 20 per cent of the calcium and magnesium were bound to macromolecules that did not pass ultrafilters. Complexing of the latter two metals by small molecules has not been investigated.

*Inorganic cations and insect activity.*—The high potassium and magnesium and low sodium levels found in the hemolymph of many insects raise questions concerning neuromuscular function which have been intensively investigated [see Hoyle (146)]. These cannot be discussed here, but some interesting postulated relationships of hemolymph ion levels to insect behavior, proposed by Hoyle, are worth mentioning. In locusts, the blood potassium level may fall by as much as 50 per cent during overnight starvation, resulting in a significant rise in muscle resting potential. The consequent heightened muscular irritability leads to the greater activity of starved animals and is believed to be one of the factors setting off marching behavior (101, 142). During molting in *Locusta* nymphs, on the other hand, there is a temporary rise in K and fall in Na in the blood, a change believed to be responsible for their quiescence during the process (145).

*Inorganic anions and ionic balance.*—Since Buck's review (42), chloride has been determined in the hemolymph of *P. maritimus* (192), *Sialis* [also bicarbonate (248)], *Carausius* (= *Dixippus* auctt.) *morosus* Brunner (232), *Periplaneta* (10), *A. polyphemus* (52), and 10 other species (97). The "chloride indices" of Duchateau *et al.* (97) show that, among the insects listed, the proportion of total inorganic cation equivalents neutralized by chloride ranges from 7.2 to 78.4 per cent, the majority of species falling in the lower part of this range; this contrasts with the situation in vertebrates, where chloride is always the predominant anion. The contributions of various anions to the balance in insects have been discussed by Buck (42); recent work on organic acids and phosphates (see below) confirms their importance. The free amino acids in hemolymph frequently make a net contribution to cations rather than anions, since aspartic and glutamic acids (in contrast to their amides) occur in low concentration, and one or more of the basic amino acids is generally abundant (see below).

*Trace elements.*—A number of trace elements have been revealed in the

ash of various tissues of the silkworm, *B. mori* (120). Copper in the hemolymph (mostly bound in tyrosinase) has been determined quantitatively, ranging from 0.9 to 1.9 μg./ml. during the fifth instar (162).

## Free Amino Acids

One of the most characteristic features of insect hemolymph is the high level of free amino acids. First shown by Tsuji (282) and confirmed by determinations of amino N [see Buck (42); also Koenig (168) for *Apis mellifera* Linnaeus and Drilhon (84) for *Macrothylacea rubi* (Linnaeus)], the phenomenon has received closer attention with the advent of specific micromethods for amino acid analysis. Whereas various vertebrates have about 50 mg. of free amino acids per 100 ml. of plasma and several non-hexapod invertebrates have less than 100 mg., the concentrations in a number of insect species examined in Florkin's laboratory range from 293 to 2430 mg./100 ml. (93).

*Qualitative analyses.*—Paper chromatography has been used to identify the free amino acids in the blood of a considerable number of species. In addition to the results tabulated by Buck (42) [see also Chauvin (53); Hinton (138)], such analyses have been done on *B. mori* (151, 167, 304), *Antheraea pernyi* Guérin (108), *M. rubi* (84), *Galleria mellonella* (Linnaeus) (30), *T. molitor* (200), *Anomala orientalis* Waterhouse (230), *Acyrthosiphon pisum* (Harris) (11), and others [8 spp., Drilhon (82); 9 spp., Auclair & Dubreuil (13); 5 spp., Clark & Ball (64)]. Amino acids in *Bombyx* have also been analyzed by high-voltage paper electrophoresis (81).

By these methods, all of the approximately 20 amino acids known to be constituents of proteins have been demonstrated in insect blood, though the proprotions vary widely, and some are not detectable in certain species or stages. Other related compounds that have been found include: β-alanine, ornithine, α-aminobutyric acid, and taurine (commonly present), cystathionine [in *Bombyx* (170)], 3-hydroxykynurenine [in *Bombyx* (149, 197)], and γ-aminobutyric acid and *S*-methyl cysteine [in *Prodenia* (150)]. Of unusual interest is the apparent presence of D-alanine in the blood of the milkweed bug, *O. fasciatus*, but not in milkweed seeds, the food of this species (14). It would be desirable to have this report, which is based on reactions of chromatogram spots, confirmed by isolation of the compound.

*Quantitative analyses.*—A number of quantitative analyses of free amino acids in insect blood have also now been done. Most active in this field have been Duchateau, Florkin and co-workers, who have used microbiological assay to determine 15 amino acids in dialysates of hemolymph from some 35 species of insects, all but six of which are Lepidoptera [results published in many short papers are compiled and discussed by Duchateau & Florkin (93) and also in part by Florkin (112, 113)]. In a few cases only, analyses were done with and without prior hydrolysis, thus estimating glutamine and asparagine. Serine and tryptophan were not determined. Fukuda *et al.* (118, 119) have also used microbiological assay for the amino acids in

*Bombyx* hemolymph. Quantitative paper chromatography has been used to determine amino acids in the blood of *D. melanogaster* (60, 268), *Anagasta* (=*Ephestia* auctt.) *kühniella* (Zeller) and *Chaoborus* (=*Corethra* auctt.) *plumicornis* (Fabricius) (60), *B. mori*, *G. mellonella*, and *Diprion hercyniae* (Hartig) (299), *Calliphora augur* (Fabricius) (123), and *P. eridania* (150), while the ion-exchange chromatographic technique of Moore & Stein has recently been applied to blood amino acids of *Bombyx* (169, 171).

Among the species examined by Duchateau & Florkin, the representatives of the more primitive orders tend to have lower total amino acid levels than the more specialized insects. The proportions of the individual amino acids are fairly characteristic for a species at a given stage (the analyses on *B. mori* in several laboratories are in quite good agreement) but vary widely among species. There are certain common features, however; thus, almost all insects have high concentrations of glutamine and of proline and of one or more of the basic amino acids, arginine, lysine, and histidine. The use of the free amino acid patterns of whole insects as a taxonomic character has been suggested [e.g., Clark & Ball (64); Micks & Gibson (204)]. In using amino acids in this way, however, the factors that influence their concentration within a species must not be ignored.

*Relation to food intake.*—Auclair (12) has shown that feeding various amino acids to *Blatella germanica* (Linnaeus) alters the blood levels of both the substance ingested and others metabolically related. In *P. eridania*, the presence of *S*-methyl cysteine is dependent upon dietary supply of *S*-methyl cysteine sulfoxide (in kale); transfer to a diet of potatoes eliminates this amino acid from the hemolymph and alters the levels of some others (150). In *Sphinx ligustri* (Linnaeus), the amino acid patterns of caterpillars fed on different plants do not seem to differ more than those of several collections from one host (92). During periods of enforced starvation, amino nitrogen may remain approximately constant [*Celerio euphorbiae* (Linnaeus) (206)] or even rise [*Popillia japonica* Newman (196)], while the quantitative pattern of amino acids changes somewhat [*Aeschna* sp. (93); *Bombyx* (171].

*Changes with development.*—Quantitative differences in amino acids have been observed between larvae and pupae of *Calliphora* (123), *Bombyx* (113, 169, 171, 299), and other Lepidoptera (93), and large changes occur in *C. euphorbiae* and *S. ligustri* during development of the adult from the diapausing pupa (113). While these are undoubtedly related to the metabolic changes associated with metamorphosis, it does not yet seem possible to give any more specific interpretations. Silkworms taken during the last larval molt show elevated tyrosine, presumably in preparation for tanning of the new cuticle (299).

Changes in hemolymph amino acid and peptide concentration during insect development have also been studied in relation to the analysis of gene action. Thus, several genotypes of *A. kühniella* show quantitative differences [Chen & Kühn (61)], and lethal mutant larvae of *Drosophila*, studied in Hadorn's laboratory, accumulate amino acids and peptides

abnormally, presumably in reflection of deranged protein metabolism (103, 124, 268).

*Relation to silk production.*—The amino acids in silkworm hemolymph have received particular attention in relation to the question of the source of materials for silk synthesis. Florkin and co-workers (4, 36, 38, 96, 98, 99, 244) have followed changes in concentration during the fifth instar and the pupal period: most amino acids rise, then fall, in the fifth instar and rise again at pupation. Histidine and lysine, however, fall during pupation. The effect of experimental extirpation of the silk gland was tested (see also 119) and found to cause accumulation of extraordinary levels of several amino acids that are constituents of silk or their metabolic precursors (e.g., glutamine, threonine, and glycine, but, surprisingly, not alanine and serine). Injected radioactive amino acids are efficiently incorporated into fibroin (35, 39, 251). It is concluded that free amino acids in hemolymph are major precursors for silk synthesis and that the normal silkworm maintains a delicate balance between secretion of amino acids into the hemolymph (both from food and from other sources) and withdrawal from it. Sham operations showed that experimental injury has a significant enhancing effect upon the levels of certain amino acids.

*Changes in disease.*—Changes in hemolymph amino acids have also been observed in insects in various pathological states: *Bombyx* infected with nuclear polyhedrosis (88), flacherie (90), and muscardine (167), and bees infected with the mite *Acarapis woodi* (Rennie) (168).

### Other Non-Protein Nitrogenous Compounds

Of the total non-protein nitrogen of insect blood, only a portion [on the average 35 to 65 per cent (42, 299)] is attributable to amino acids. The other compounds most often reported in this fraction are the end-products of nitrogen metabolism: uric acid, allantoin, allantoic acid, urea, and ammonia. Uric acid, the principal form of excretory nitrogen in insects, is universally present; its identity in *C. euphorbiae* has recently been confirmed by spectrophotometric and chromatographic methods (136). The levels are commonly close to saturation (6.5 mg./100 ml. in water), and, when this is exceeded, crystalline granules may circulate, as in the *Popillia* pupa (193) and *Prodenia* (6). During starvation, uric acid levels may fall somewhat (6, 194). Removal of the silk glands leads to elevated uric acid in *Bombyx* (47). Allantoin has been reported in hemolymph [*A. pernyi* (178); *Popillia* (194)] and is presumably derived from uric acid by uricase, an enzyme that is present in some species at some stages of development (41, 234, 239). Further degradation by allantoinase yields allantoic acid, which is found in the hemolymph of *Bombyx* (199) and some, but not all, other Lepidoptera (234). Conversion of allantoic acid to urea apparently does not occur (234); urea, however, is frequently present in the hemolymph [see Buck (42); also Ludwig (193) and Ludwig & Cullen (194) for values in normal and starved *Popillia*], probably as a product of the action of arginase, which some species

do possess (121, 165). Ammonia is a significant end-product chiefly in aquatic forms; even in these, however, the concentration in the hemolymph is low [0.5 mg./100 ml. in *Sialis* (261)], possibly because of "storage" as the amide group of glutamine (188).

Other non-protein nitrogenous substances reported in hemolymph are basic phosphates and amino sugars (see below). Unidentified ninhydrin-reactive substances are commonly reported; some disappear upon acid hydrolysis and are presumably peptides (61, 171, 299). Peptides appear to be abundant in *Drosophila* (124, 268), though quantitative analyses in absolute units have not been reported. The rather small release of amino acids other than glutamic and aspartic by hydrolysis of deproteinized hemolymph of several Lepidoptera suggests that, in these cases, peptides are quantitatively minor (113, 169). Indeed, most of the substances discussed above occur in amounts that are small in relation to the unassigned non-protein nitrogen, indicating that the major nitrogenous components of hemolymph remain to be identified.

## Organic Acids

As early as 1909, Tsuji (282) described extraordinarily high levels of organic acids, identified as citric, malic, succinic, and lactic, in larval *Bombyx* hemolymph. Apart from citric acid (see below), there seems to be no published attempt to confirm this report up to the present time. Indeed, there have been very few analyses of organic acids in the blood of any insect.

The only comprehensive work is that of Levenbook on hemolymph of third-instar *Gasterophilus* larvae. This hemolymph contains the following, expressed as m.eq./l.: succinate, 41 (189); malate, 52 and fumarate, 9 (212); citrate, 7 and lactate, 1 (181); and $\alpha$-ketoglutarate, 13 [(184); cf. Buck (42, p. 163)]. Collectively, these make up some 50 per cent of the anion titer.

In *Bombyx* larval hemolymph, several $\alpha$-keto acids have recently been identified as their dinitrophenylhydrazones. Murthy & Sreenivasaya (208) reported pyruvic and three unknowns; Fukuda and co-workers (116, 117) found no pyruvic, but they did find glyoxylic at 4 to 7 m$M$ and $\alpha$-ketoglutaric, oxaloacetic, and acetoacetic in smaller amounts. In *A. pernyi*, pyruvic acid has been reported at 23 to 31 m$M$ (48). Preliminary results from a study of organic acids in hemolymph by ion-exchange chromatography indicate the presence, in one or more developmental stages of *Hyalophora cecropia* (Linnaeus), of citrate, malate, succinate, fumarate, $\alpha$-ketoglutarate, pyruvate, and several unknowns, the total amounting to 25 to 35 m.eq./l. (245).

Citrate seems to occur in insect blood generally. It is present in larval *Rhodnius prolixus* Stål (2.3 m$M$) and adult *Tenebrio* (5.0 m$M$) (227), and, recently, Levenbook (186) established its presence, frequently at high concentrations, in hemolymph of 16 species. The greatest amount found was in the *Bombyx* larva, which contained 32 m$M$ (96 m.eq./l.!), a value not far from that reported by Tsuji 50 years earlier. Clearly, organic acids in hemolymph need further study.

### Compounds of Phosphorus

It is well established that insects are exceptional among animals in the high content of phosphates in their plasma. That only a minor fraction of this is inorganic orthophosphate seems to have been noted first for *B. mori* (25, 27) and has been confirmed for a number of other species [see Buck (42); also Heller (130)]. Most of the organic P is acid-soluble, only small amounts falling in the phospholipid and phosphoprotein fractions (139, 160, 163, 181, 299). The application of controlled hydrolysis showed the acid-soluble P to include fractions of differing stability, the majority being difficult to hydrolyze (130, 160, 163). Fractional precipitation of barium salts led to presumptive identification of several compounds from hemolymph of blowflies [Hopf (139)] and *A. pernyi* [Smolin (259)]; this method, however, is not reliable when used on complex unknown mixtures. Recently, in the reviewer's laboratory, ion exchange chromatography has been used to fractionate hemolymph of several species of Lepidoptera, and a number of P compounds have been identified by chromatographic, chemical, and enzymic means (294, 297, 298, 301). Of the acid-soluble phosphates in *H. cecropia* (total concentration 30–40 m$M$), the major components are: orthophosphate (3 to 10 mM), $\alpha$-glycerophosphate (6 to 18 m$M$), phosphorylcholine (2 to 14 m$M$), and phosphorylethanolamine (0.6 to 5 m$M$). The last-mentioned, as well as glycerophosphoethanolamine, has also been determined in *Bombyx* hemolymph by Kondo & Watanabe (171). *Bombyx* differs from *H. cecropia* and several other Lepidoptera in that $\alpha$-glycerophosphate is virtually lacking, whereas sorbitol-6-phosphate is abundant and glucose-6-phosphate is also present. A nucleotide fraction incorrectly reported as ATP (294) has proved to comprise several uridine diphosphate sugar derivatives (51). The occurrence of a basic phosphate that has the extreme acid lability characteristic of phosphoarginine [previously reported in *C. euphorbiae* hemolymph by Heller (130) and Heller & Moklowska (134)] has been confirmed (50), but this compound remains to be isolated.

In *C. euphorbiae* hemolymph, Heller has shown that inorganic phosphate rises greatly during the pupal diapause and falls again as the adult develops (128, 134); he incorporated this observation into a theory of metabolic control in diapause (131). Characteristic changes in hemolymph phosphorus fractions in different developmental stages also occur in other species (152, 160, 163, 259, 294).

Experiments with $P^{32}$ indicate that the presence of cellular tissues is necessary for biosynthesis of the blood phosphates, and some measurements of incorporation rates have been made (159, 294). The reason for selective accumulation of certain phosphates in the plasma is unknown [see discussion by Wyatt (294)].

### Carbohydrates and Related Substances

*Reducing substances.*—When the classical methods for determination of sugars by reducing power (reduction of cupric ions or ferricyanide) are

applied to insect blood, high values are invariably obtained. As early as 1924, however, it was recorded (137) that only a small fraction (in this case some 25 per cent) of the reducing material in *Bombyx* hemolymph was sugar fermentable by yeast; this observation was subsequently confirmed for many species [see Buck (42); also Todd (277)] and is consistent with the failure to prepare glucosazone from silkworm extracts (285) and with the low levels of reducing sugars detectable by paper chromatography (299). Nevertheless, designation of the reducing compounds as glucose persists in some recent papers (286). The reducing substances of lepidopteran hemolymph differ from glucose in that they react with ferricyanide in the cold (133, 174). They probably comprise, in fact, a mixture of substances such as uric acid, ascorbic acid, amino sugars, tyrosine, and, possibly, dihydroxyphenylalanine and other phenols (133, 134, 135, 174, 196). Sulfhydryl compounds may contribute, although high -SH has never been demonstrated in hemolymph. In pupae of *C. euphorbiae*, the reducing power seems to be largely accounted for by tyrosine (133). It is noteworthy that in *Bombyx* the reducing substances rise sharply at each molt (74, 174), at which time blood tyrosine also increases (299), and a rise in any substances associated with breakdown and resynthesis of the cuticle would be expected.

*Monosaccharides.*—In adult bees, free reducing sugars do occur at the exceptional levels of 1 to 4 per cent of the hemolymph; these levels have been established by fermentation and by chromatography (69). The latter technique indicates that 20 to 40 per cent of the sugar is fructose, the remainder glucose. High levels (up to 2 per cent) of glucose are also found in *C. euphorbiae* pupae exposed to low temperature (132). Lower levels of glucose are found in hemolymph of *Phormia regina* (Meigen) [larva 70 to 125 mg./100 ml., adult up to 600 ml./100 ml. (102)], and very low levels (from 0 to 50 mg./100 ml.) are found in the *Gasterophilus* larva (181), larvae and pupae of *B. mori* (299), larvae of *G. mellonella* and *Diprion hercyniae* (Hartig) (297), and pupae of *H. cecropia* and *Samia cynthia* (Drury) (295). In *Gasterophilus*, fructose is relatively abundant [up to 280 mg./100 ml. in the larva, less in the pupa (179, 181)], but this is exceptional; no appreciable amounts were found in three other insects examined by Levenbook (179), and only traces, in *Bombyx* (299).

*Trehalose.*—The virtual absence of reducing sugar from the blood of various insects stands in contrast to Heller's (129) finding of 4 per cent of "nitrogen-free extractives" in hemolymph of *C. euphorbiae* pupae and to the presumable need of active insects for a mobile energy source. This inconsistency was resolved by the recent demonstration that the principal blood sugar of many, if not most, insects is the non-reducing disaccharide trehalose. This was foreshadowed by (*a*) the report of "polysaccharide below glycogen" in bee larvae (238), (*b*) the observation that acid hydrolysis releases much fermentable sugar in *Bombyx* hemolymph (174), and (*c*) the report of much unidentified anthrone-reactive material in the hemolymph of several species (299). In 1956 and 1957, reports from three laboratories,

with the support of rigorous chemical identification (297), showed trehalose to be the major blood sugar of various insects [Evans & Dethier (102); Howden & Kilby (140); Wyatt & Kalf (296)].

The levels of trehalose found in different species and stages vary tremendously. Values over 1000 mg./100 ml. are common, the highest yet reported being 6554 mg./100 ml. in a larval solitary bee (94). Some information is accumulating on changes with stage of development. In *Bombyx*, the fifth-stage larva has 250 to 600 mg./100 ml., and the level falls from the time of cessation of feeding to a minimum at pupation, then rises again during adult development (95). In *H. cecropia*, the pattern is similar: from about 2000 mg./100 ml. in the mature larva, trehalose falls to some 600 mg. in the early pupa, 150 to 300 mg. during diapause, and then rises again at initiation of adult development (16, 295, 297). In *C. euphorbiae*, however, the pattern is very different: the feeding larva apparently lacks trehalose, this sugar first appears in the blood at the cessation of feeding, then rises during pupation to about 1500 mg./100 ml., is maintained around 1000 mg. during diapause, and falls with development of the adult [Mochnacka & Petryszyn (205); cf. Duchateau & Florkin (94) for *Calephila elpenor* (Linnaeus)]. In *P. regina* also, the larva lacks trehalose whereas the adult contains large amounts (102).

Blood trehalose levels respond strikingly to nutritional state. In *Schistocerca gregaria* (Forskål), radioactivity ingested as glucose, fructose, or mannose rapidly appears in the hemolymph as trehalose; this fact supports the theory that conversion of sugars to trehalose facilitates their diffusion through the gut wall [Treherne (279 to 281)]. In *P. regina*, blood trehalose levels rise after feeding and fall during flight (102, 147). Marked fall during several days of starvation has been observed in *Bombyx* (94). It seems clear that trehalose has the role of a mobile, rapidly synthesized and utilized nutrient reserve, but the functions of different tissues in its metabolism and the nature of the regulatory mechanisms are questions still awaiting investigation.

*Amino sugars and derivatives.*—In hemolymph of *T. molitor*, Marcuzzi (200), on the basis of paper chromatography and colorimetric methods, has reported glucose at 122 mg./100 ml. and glucosamine in "notably greater quantity." In *Bombyx*, ion exchange chromatography shows a small peak of glucosamine (171), while colorimetric methods failed to detect hexosamine but indicated small amounts of N-acetylhexosamine (299). A recent study of glycoproteins indicates that in *P. americana*, approximately half the carbohydrate in hemolymph is thus bound, and upon hydrolysis glucosamine, galactosamine, mannose, galactose, smaller amounts of glucose, and a pentose are liberated [Lipke *et al.* (191)]. The quantities fluctuate during the molting cycle. N-Acetylgalactosamine and N-acetylglucosamine, as well as galactose and glucose, have been identified in *H. cecropia* plasma as their uridine diphosphate derivatives, presumably precursors of oligo- and polysaccharides (51). These fragmentary observations suggest that systematic

investigation of free and bound amino sugars in insect hemolymph would be fruitful.

*Glycogen.*—Some apparent glycogen has been reported in the hemolymph of *Bombyx* (249, 297) and some other insects [see Buck (42)]. The widely discrepant values obtained for *Bombyx* by different methods suggest that polysaccharides other than glycogen are contributing. Material interfering in glycogen analysis occurs also in *Gastrophilus* blood (181). Glycoproteins are one possible source of such trouble.

*Glycerol.*—An unexpected solute recently found at phenomenal levels in some insects is free glycerol. The report of glycerol in concentrations up to 3 per cent in hemolymph of diapausing *H. cecropia* pupae (298, 300) led to the suggestion by Salt of a possible role in cold-hardiness (241; see also pp. 55–74). This was supported by its demonstration in a number of other species, including *Bracon cephi* (Gahan), in which glycerol (in whole insects) may attain 25 to 30 per cent and is correlated with the supercooling point (242), and carpenter ants, in which production of glycerol follows exposure to low temperatures (91). Protection of isolated insect hearts against cold injury by glycerol at 5 per cent or more has been demonstrated (291). Nevertheless, not all cold-resistant insects accumulate glycerol; even some saturniid pupae closely related to *H. cecropia* contain little or none (300). Accumulation of glycerol [and of sorbitol in the *Bombyx* egg; Chino (62)] during diapause as reduced products of carbohydrate metabolism is also of interest in relation to problems of metabolic control (300).

## Lipids

In addition to those cited by Buck (42), a few determinations of insect hemolymph lipids have been recorded; these indicate substantial levels. In hemolymph of *C. euphorbiae* pupae before winter, total fat is 1975 mg./100 ml.; after winter total fat is 830 and cholesterol 25 mg./100 ml. (129, 134). The adult *Hydrous* (= *Hydrophilus* auctt.) *piceus* (Linnaeus) has total fatty acids 550 mg./100 ml., unsaponifiable substances 220 mg./100 ml. (86), and the *P. japonica* larva averages 420 mg./100 ml. total lipids (196). The *Bombyx* pupa has 10 to 27 mg./100 ml. of lipid P (160). On the basis of Sudan Black staining of electrophoretically separated bands, lipoproteins have been reported in the silkworm and other insects by several authors (166, 252, 287). Although the chemical characterization of these lipids would be feasible by modern chromatographic methods, no such investigation has yet been published, nor has there been any report on their metabolism.

## Pigments

Since insect pigments, including the new class of aphid hemolymph pigments, the aphins, have been reviewed recently by Cromartie (68), only a few supplementary points will be mentioned.

Although there has been some skepticism (68), the occurrence of $\alpha$-carotene (in contrast to the ubiquitous $\beta$-carotene) in insect hemolymph

seems to have been established by the careful spectrophotometric work of Chefurka [(54); Chefurka & Williams (55)] on the compound isolated chromatographically from *H. cecropia*. Pupal hemolymph of this species also contains taraxanthin, the two carotenoids totalling some 2.5 mg./100 ml. The presence of chlorophylls, mentioned in an abstract (55) and cited by Buck (42), is not established in the fuller report (54).

Chefurka [(54); Chefurka & Williams (56)] also measured riboflavin and flavin nucleotides in *Hyalophora* hemolymph and showed these to be much higher in the diapausing pupa (total flavins 3.8 mg./100 ml.) than during development. The hemolymph riboflavin levels of two strains of *B. mori* during metamorphosis have been reported by Eguchi (100). Other work on fluorescent substances is that of Drilhon and co-workers (83, 87, 88), who report the presence of folic acid, fluorescyanine, flavones, and flavins in normal and pathological *Bombyx* hemolymph. It would be desirable to have these identifications, based on paper chromatography, confirmed by more rigorous methods.

Pigmentation has also been studied from the genetic point of view. Kikkawa (164), in a review on biochemical genetics of the silkworm, discusses the relations between genes and selective permeabilities of intestinal and silk gland walls; these relationships influence the pigments accumulating in the hemolymph and cocoon. Earlier work on this subject is reviewed by Timon-David (275), and a recent contribution reporting resolution of flavonoid pigments is that of Fujimoto *et al.* (115). The sex difference in hemolymph color of many Lepidoptera described many years ago by Geyer (122) has been analyzed genetically in *Choristoneura* species by Stehr (262) and was shown to be the result of balance between autosomal and sex-linked loci concerned with the accumulation of blue bile pigment.

## Proteins

*Characterization of hemolymph proteins.*—During the past few years, the previous neglect of insect blood proteins has been replaced by a flush of experiments, chiefly using the simple techniques of electrophoresis on filter paper or, most recently, on starch or agar gel. This work is summarized in Table I. The standard conditions for analysis of human plasma (pH ca. 8.6, ionic strength 0.05 to 0.1) have generally been used and seem to be suitable for hemolymph proteins also. Electrophoresis on paper has resolved only a small number of protein bands (1 to 5 in various species), generally migrating with velocities comparable to those of the mammalian plasma globulins. The designation of these proteins as albumin, $\alpha$- and $\beta$-globulin, and so on, solely on the basis of their mobility (85, 148), is quite unjustified, as pointed out by Denucé (78). The agar and starch gel techniques, in which the separation apparently depends on interactions with the medium in addition to pure electrophoretic mobility [Smithies (258)], give sharper bands in greater number. The patterns obtained are, to a large extent, characteristic of the species, and their use for taxonomic purposes has been suggested (22, 34, 243, 265). It

TABLE I

ELECTROPHORETIC SEPARATION OF HEMOLYMPH PROTEINS

| Technique | Organism and Stage | Results | Reference |
|---|---|---|---|
| Moving boundary | *Schistocerca* (adult) | 3 peaks; change with clotting | 31 |
| | *Culex*, 3 Lepidoptera | Several indistinct peaks | 65 |
| | *Hyalophora* (adult) | 3 main peaks, several lesser; sexes differ | 271 |
| | *Bombyx* (larva, pupa) | 3 main peaks | 222 |
| Paper | *Periplaneta* (larva, adult) | 3 main bands; differ with stage | 65, 263 |
| | *Periplaneta* (larva, adult) | 5 bands, including glyco- and lipoproteins; differ with molting | 252, 253 |
| | *Curculio* spp. (larva) | Up to 5 bands in patterns apparently correlated with species | 34 |
| | *Melolontha, Neodiprion, Porthetria* (larva, adult) | 2 to 4 bands; isoelectric points (*Melolontha*) 6.5 and 6.8 | 172 |
| | *Drosophila* (larva) | 2 bands; normal and lethal types differ | 57, 293 |
| | *Culex* | 1 band; autogenous and anautogenous forms differ | 58 |
| | *Periplaneta, Blatta, Oncopeltus, Pleocoma, Lichnanthe, Phormia, Smerinthus* (♀ adult) | 2 to 4 bands in patterns characteristic of species | 265, 266 |
| | *Galleria* (larva) | | 76 |
| | *Bombyx* (larva) | 2 to 3 bands, including glyco- and lipoprotein; differ with stage, molting, sex, and diseases | 1, 2, 78, 85, 148, 166, 236 |
| Agar gel | *Tenebrio, Dytiscus, Galleria, Phragmatobia Macrothylacea* (larva) | Bands more numerous, better separated, and in different sequence than on paper | 80 |
| | Triatomidae, ticks | Up to 11 bands, in specific patterns; independent of age, sex, food, and infection | 22, 243 |
| Starch gel | *Drosophila* (larva) | 7 bands; differ in normal and lethal types | 58 |
| | *Culex* (larva) | 4 bands; quite distinct from *Drosophila* | 58 |
| | *Periplaneta, Vespula, Malcosoma,* and others (larva, pupa, adult) | 5 to 15 bands; increase with development and differ with species | 287 |
| | *Galleria, Bombyx* (larva) | Up to 11 bands; increase with development and differ with species | 77, 78, 79 |
| | *Hyalophora* (larva, pupa) | Bands possess enzyme activities; differ quantitatively with stage and sex | 176 |

appears that they may have real value for this purpose, especially in a microgel technique similar to that of van Sande & Karcher (243), provided the nutritional and developmental state of the insect is adequately controlled. Differences have even been observed between normal and lethal genotypes of *Drosophila* (293) and between autogenous and anautogenous *Culex* (59).

By way of chemical characterization of hemolymph proteins, the zone-

electrophoretic methods have contributed the observation that certain bands stain with selective reagents, indicating glycoproteins and lipoproteins (2, 166, 252, 287). Siakotos (252) has also obtained reactions for phospholipid and sterol in protein bands from *P. americana* plasma.

By far the most intensive work on fractionation and physical characterization of insect plasma proteins is that on *B. mori* reported in a recent series of papers by Oda and co-workers. Both in the ultracentrifuge and in the Tiselius electrophoresis apparatus, unfractionated larval hemolymph gave three main components. The fastest sedimenting was lacking in the pupa (222). The fastest and slowest components, purified by a procedure involving precipitation and ultracentrifugal sedimentation, gave the physical constants presented in Table II (213). The sedimentation constant of component *3* is in

TABLE II

PHYSICAL CHARACTERIZATION OF HEMOLYMPH PROTEINS OF LARVAL *BOMBYX MORI**

| Component | $S_{20w}$ | $D_{20w}$ | $M$ | $f/f_0$ | Axial ratio† |
|---|---|---|---|---|---|
| *1* | 2.8 *S* | $1.61–1.73\times10^{-7}$ | 160,000 | 3.45 | 70 |
| *3* | 17.4 *S* | $2.64–2.68\times10^{-7}$ | 630,000 | 1.39 | 7.3 to 8.4 |

* Oda (213).

† Assuming no hydration.

fair agreement with that of 16 *S* found in an earlier analysis of *Bombyx* blood (177), and it is interesting that *H. cecropia* blood also contains a component of very high molecular weight [see Buck (42, p. 171)]. In further experiments, Oda found that the ultracentrifugal and electrophoretic patterns and the average molecular weight found by light-scattering were highly sensitive to changes in pH and other conditions; this sensitivity indicates the association and dissociation of the proteins (214 to 216). Fractionations by salting-out, heat denaturation, and chromatography on calcium phosphate were also tested, and some success was achieved with the last two (217 to 221). The middle sedimenting component (7 *S*) was the least stable and could not be purified.

The blood proteins of *H. cecropia* have been studied as antigens by Telfer (271, 273), who used rabbit antiserum in an agar-diffusion technique. Up to 9 bands of precipitate were observed, of which 7 were followed quantitatively through metamorphosis. All increased in amount during the last larval instar, changed little in diapause, and decreased during development of the adult, but the precise patterns differed characteristically.

*Metabolism of hemolymph proteins.*—Many workers concur that the content of protein in an insect's hemolymph fluctuates much more widely than that of free amino acids and other non-protein nitrogen. The pattern during development of several Lepidoptera is essentially similar: a severalfold in-

crease in protein concentration during larval life, at first gradual, then rapid in the last instar; little change during spinning and the first part of the pupal period; then a sharp fall during development of the adult [*B. mori* (27, 110, 111, 299); *C. euphorbiae* (134); *S. cynthia* (89); *A. pernyi* (71); *H. cecropia* (54)]. Similar changes take place in *Popillia japonica* (193). Taken together with the decrease in volume of hemolymph during adult development, this phenomenon suggests that the hemolymph proteins serve as an important source for synthesis of proteins of the adult [cf. Heller (129)]. Selective direct uptake of several blood antigens (and injected foreign proteins) into developing oocytes of saturniids has been demonstrated by Telfer (271, 272).

The importance of hemolymph protein as a reserve is also illustrated by changes during starvation. Thus, when *C. euphorbiae* (134, 206), *B. mori* (27), or *S. lutaria* (19) is subjected to enforced starvation, hemolymph protein falls markedly while non-protein N remains unchanged. This presumably reflects hydrolysis of protein to maintain amino acids and thus osmotic pressure [Beadle & Shaw (19); Buck (43, p. 201)]. A somewhat different picture, however, appears in *P. japonica;* here, prolonged starvation leads to substantial rise in amino N and total non-protein N, while the protein level remains unchanged (196).

These changes contrast with those accompanying production of silk, for which hemolymph proteins are little utilized, if at all [Denucé (78); Florkin (111); cf., however, Demjanovskii & Filippovich (71)], whereas free amino acids are efficient precursors (see above).

Little is known of the sites and mechanisms of these processes. Injected methionine-$S^{35}$ is incorporated into hemolymph proteins of larvae and nondiapausing pupae of *A. pernyi* at rapid rates comparable with those of other tissues (72). In diapausing *H. cecropia* pupae, incorporation of glycine-$C^{14}$ is slow but is greatly stimulated with initiation of development or in response to cuticular injury (274). Shigematsu (250) has presented evidence that hemolymph proteins of the *Bombyx* larva are synthesized in the fat body by demonstrating, during incubation of fat body *in vitro*, net increase in protein and secretion of proteins electrophoretically resembling those of the hemolymph. The experiments of Sissakian & Kuvaeva (256), which have been interpreted as protein synthesis in the hemolymph itself (250), were in fact done with "coelomic fluid" obtained by crushing whole pupae.

*Proteins in clotting.*—This process, which varies greatly among different insects and may involve both hemocytes and plasma proteins, has been reviewed recently by Hinton (138) and by Wigglesworth (290). Some measure of the involvement of plasma proteins is indicated by the fact that in *S. gregaria*, out of a total of 770 mg./100 ml. of plasma protein N, 420 mg. are rendered insoluble during clotting (31). The clotted protein comes entirely from the middle of three electrophoretic bands. *Periplaneta* also shows changes in the electrophoretic pattern as a result of clotting (253).

*Phenol oxidase in hemolymph.*—The darkening of insect hemolymph upon exposure to air as a result of the action of the enzyme phenol oxidase (tyro-

sinase) was observed by the earliest workers with this fluid and has been a subject of interest and speculation ever since. Nevertheless, insect blood tyrosinase has not yet been extensively purified nor characterized, and the nature of its biological role and the regulation of its activity remain obscure and controversial. Phenol oxidase has been discussed in recent reviews from the point of view of comparative biochemistry by Mason (203) and in relation to cuticular hardening by Wigglesworth (289), Richards (237), and Dennell (75), and only a few contributions concerning this enzyme in hemolymph can be considered here.

Changes in activity of tyrosinase during development of *Bombyx* have been followed manometrically, using whole hemolymph as the enzyme, by Nittono (209) and Ito (156, 157). With tyrosine and catechol as substrates, there is a fall in activity prior to both larval and pupal molts, followed by a sharp rise at each molt. If pupation is induced precociously by ligation of fourth-instar larvae, the pattern is one that is characteristic of pupation (210). When aqueous extract of blood acetone powder is used as the enzyme, however, the magnitude of these changes is much less (157). In addition, when blood is diluted with distilled water, its tyrosinase activity increases substantially during several hours of standing. Ito has attributed these results to a labile inhibitor. A strain that has very low tyrosinase activity is believed to possess less enzyme and more active inhibitor (158). Ohnishi, on the other hand, has shown that tyrosinase occurs in *Drosophila viridis* Sturtevant and *Musca domestica vicina* Macquardt as a proenzyme which can be activated by a protein activator, and, on the basis of a few experiments with *Bombyx* hemolymph, he believes that a similar system exists there (223 to 226). In other recent contributions, the inhibition and some other characteristics of *Bombyx* and *Drosophila* tyrosinase have been described (155, 223, 240).

The importance of phenol oxidase for insects is generally considered to be in the production of quinones for sclerotization of the cuticle [although Dennell (75) would restrict this to melanization]. The activity fluctuations mentioned above suggest a role in molting; yet, it is not explained why the enzyme should be so abundant in the circulating fluid as well as in the integument nor why blood tyrosinase should be as high in the middle of the fifth instar as at the time of the pupal molt (157, 209). Nittono & Takeshita (211) have shown that cuticular injury causes immediate rise in blood tyrosinase, and it seems possible that the enzyme may have a role in connection with the responses of insects to injury, which are known to include liberation into the blood of substances stimulatory to metabolism (125).

*Other enzymes.*—A considerable number of enzymic activities have been reported in insect hemolymph. The earlier work, chiefly on carbohydrases, proteases, and lipases of more or less defined specificity, has been reviewed [Chauvin (53); Timon-David (275); Yamafuji (303)]; in addition, Arvy & Gabe (7) have recorded the presence and absence of several enzymes in the

blood of a number of insect species, and the presence of phosphatase (153, 181, 294) and transaminase (21, 24), among others, have been recorded in certain species. Kikkawa (164) has discussed the genetic determination of blood amylase in *Bombyx*. Laufer (176) has shown various hydrolytic enzyme activities to be associated with the electrophoretically separable protein bands from *H. cecropia* hemolymph. In most cases, the distribution of enzymes between plasma and tissues has not been determined, and there is no evidence for their function as hemolymph components; one suspects rather that their presence in the hemolymph may be the result of a certain leakage from cellular tissues, as is known to occur in mammals (305).

Three enzymes of carbohydrate metabolism occurring in *Bombyx* hemolymph have been rather thoroughly studied by Faulkner, and metabolic roles have been suggested. These are a specific hexose-1-phosphatase (104), a TPN-linked malic enzyme (105), and a TPN-linked polyol dehydrogenase which acts both on certain free polyhydric alcohols and certain phosphates (106, 107). For the last of these enzymes the concentration in larval hemolymph (based on total protein) was shown to be intermediate between that in fat body and that in gut tissue.

Of especial interest is the presence of a specific trehalase in hemolymph which also contains its substrate (140, 228). Friedman (114) has shown that trehalase in *Phormia* hemolymph acts only after dilution, possibly because of release from a naturally occurring inhibitor.

*Antibody-like substances.*—Despite the evidence of many early experiments that insects injected with bacteria can acquire immunity to a subsequent challenge [see Steinhaus (264)], the results of recent attempts to demonstrate antibodies in hemolymph by the precipitin, agglutinin, complement fixation, antitoxin, and antigen retention tests have been almost uniformly negative (23, 40, 173, 267). Recent experiments by Briggs (40), however, confirm that the bactericidal power of the blood of various Lepidoptera is enhanced following immunization. The "immune principle" differs fundamentally from vertebrate antibodies: it has low specificity, is heat-stable, and is resistant to weak acid and alkali, but apparently is inactivated by pepsin. Stephens (267) has also confirmed that *G. mellonella* and other insects can acquire limited immunity to infection, but she has not described the role of the hemolymph. Chemical characterization of the factor found by Briggs will be of great interest.

## Conclusions and Problems

*Completeness of analysis.*—In order to reckon the extent to which present analyses encompass its constituents, some gross data on hemolymph of two of the most studied forms of Lepidoptera are compiled in Table III. A number of approximations are involved, the roughest probably being the assumption of 16 per cent N for the unidentified non-protein nitrogenous compounds. Nevertheless, in both cases, the sum of the estimated components approaches

TABLE III

SUMMATION OF COMPONENTS OF HEMOLYMPH

| Component | *B. mori* larva, mid-fifth stage | | *H. cecropia* pupa, ♀, in diapause | |
|---|---|---|---|---|
| | gm./100 ml. | Reference | gm./100 ml. | Reference |
| Na, K, Ca, Mg, Cl | 0.4 | 25 | 0.4† | 52 |
| Phosphates | 1.1 | 295 | 0.6 | 294 |
| Citrate | 0.6 | 186 | 0.2 | 186 |
| Trehalose | 0.4 | 297 | 0.5 | 297 |
| Glycerol | 0.0 | 295 | 2.8 | 300 |
| Amino acids | 1.2 | 299 | 0.9* | 54 |
| Other non-protein N | 1.9* | 299 | 2.0* | 54 |
| Protein | 1.9 | 299 | 7.1 | 54 |
| Summation of above | 7.5 | | 14.5 | |
| Total non-volatile matter | 7.8 | 299 | 15.8 | 297 |

* N×6.25.

† From values for *A. polyphemus*.

the total non-volatile matter. Many details remain to be filled in, and substances that are biologically important, although present in small amounts, doubtless remain to be discovered.

*Hemolymph and artificial media.*—A practical objective of insect hemolymph analysis is that of formulating media for experimental work with insect tissues *in vitro*. If the cells of insects are in fact in direct chemical contact with the hemolymph, such solutions should theoretically resemble hemolymph in their composition.

The question of the extent to which insect tissues may be protected from their ionic environment by a barrier membrane, however, is at present unsettled. Pipa & Cook (229), who reviewed the literature on connective tissue in insects, believe that it does provide a general barrier and that "the old concept of hexapods having an open blood stream is misleading." It may be noted, however, that the experiments giving direct evidence for such protection (141, 283, 284) have all been done with nerve tracts, which possess a layered sheath different from the connective tissue membrane of muscle and other tissues (144). Wood (292) has considered a possible selective barrier in relation to the effects of ions on *Carausius* muscle and concluded that other explanations are adequate and that the membrane probably affords no more than some slowing of penetration. Experiments on *A. polyphemus* support a similar conclusion (52).

Many solutions have been used as media for insect tissues: for a tabulation see Buck (42, p. 161); also, for nerve-muscle preparations, Wood (292) and Hoyle (143); for isolated hearts of *Chortophaga* and *S. cynthia*, Barsa

(18), of *Periplaneta*, Ludwig *et al.* (195), and of *Tenebrio*, Butz (49); for *Bombyx* fat body, Shigematsu (250); for *Carausius* Malpighian tubules, Ramsay (231); and for embryo and tissue culture of several species, the review by Day & Grace (70), also Takami (269), Trager (278), and discussion by Martignoni (202). Few of these solutions resemble hemolymph; most are closer to mammalian plasma in their inorganic content. Only in the heartbeat experiments were ion ratios systematically varied, and here a rather wide tolerance was found, although optimal Na/K ratios were in some cases higher than those of hemolymph, and Mg was found to be toxic. The tissue culture medium of Wyatt (302), on the other hand, like *Bombyx* hemolymph, contains high K and Mg. Also included, however, is a high concentration of amino acids. Unfortunately, the effect of varying the ingredients of this medium individually was not tested, and the extent of antagonism or interaction of components is not known. Such systematic tests for both tissue culture and the simpler requirements of shorter-term metabolic experiments are urgently needed.

*Interactions of hemolymph and tissues.*—It is evident that insect hemolymph, in addition to transporting nutrients and waste products between tissues, can have a significant storage function, substances being accumulated in some circumstances and utilized in others. To understand these processes, knowledge of the distribution of solutes between plasma and tissues is essential. We have little information on this, but it is clear that distributions can be very uneven and can change quickly. Thus, prepupal *C. euphorbiae* contains much trehalose in its blood but virtually none in the tissues; after pupation, the sugar is found in both compartments (205). Bricteux-Gregoire & Florkin (37) have measured the distribution of amino acids between tissues and hemolymph of larval *Bombyx:* whereas arginine is much more concentrated in the intracellular, histidine and lysine are much higher in the extracellular compartment. Again, the level of inorganic phosphate in diapausing *H. cecropia* plasma is severalfold higher than in fat body; with the beginning of adult development, this difference is much reduced (50). Such changes may be important in the regulation of metabolism.

Finally, the transport of hormones by hemolymph should be mentioned. Experiments by ligation, transplantation, and parabiosis indicate that the several endocrine agents controlling metamorphosis reach their targets via the circulation. The presence of significant amounts of growth hormone in hemolymph has been most directly shown by the tissue culture experiments of Schmidt & Williams (247). Sex cells from testes of diapausing *H. cecropia* pupae explanted into hemolymph from the same stage remain unchanged, but, in blood from developing stages, they promptly differentiate into spermatids.

Thus, the continued attention of insect physiologists to the hemolymph appears to be justified, and, with the advance of chemical knowledge, we have reached the phase when we can hope for a more precise understanding of the interactions of hemolymph and the various tissues.

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# THE ROLE OF MITOCHONDRIA IN RESPIRATORY METABOLISM OF FLIGHT MUSCLE[1,2]

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The sarcosomes of flight muscle of fast-flying Diptera and Hymenoptera have been known to cytologists for at least a century. In retrospect, it is astonishing to realize that less than a decade ago the pioneering experiments of Watanabe & Williams (106) showed that sarcosomes are the mitochondria of this highly specialized muscular tissue. Since then, insect mitochondria have become an important subject of research within several disciplines of experimental biology. For the biochemist and physiologist, mitochondria and the other morphological particulars of flight muscle afforded a first opportunity for a cytological mapping of enzyme systems which, in turn, led to experimental studies of cytoplasmic-mitochondrial interrelationships and of metabolic control mechanisms on a subcellular level.

Because of limited space, this discussion will be focused on the mechanism of mitochondrial energy metabolism and its relation to the physiological function of flight muscle. This necessitates the omission of many noteworthy papers concerned primarily with respiratory pathways in tissues other than flight muscle, especially those of Shappirio & Williams (99, 100) on wing epithelium; Pappenheimer & Williams (72) and Ito, Horie & Ishikawa on mid-gut (48); Clements (25) on fat body; and Faulkner (31) on hemolymph. This review is not intended to give a complete listing of the literature in which flight muscle was used. Instead, papers have been selected that to me seem to contribute the most significant material. By arbitrarily limiting the coverage, it is hoped that we may make intelligent use of the existing information in order to direct attention to areas requiring further study.

## Chemical Composition of Flight Muscle

Representative values for a variety of metabolites found in flight muscle at rest are summarized in Table I. Despite the fragmentary and unsatisfactory state of our knowledge of the chemical composition of insect muscle, these data provide numerous leads worthy of further consideration.

The large glycogen deposits in flight muscle, over 30 $\mu$M equivalents/gm. wet weight in the house fly [Sacktor (83)], as well as the depletion of these re-

[1] The survey of the literature pertaining to this review was concluded in March, 1960.

[2] The following abbreviations are used in this review: ADP for adenosine diphosphate; AMP for adenosine monophosphate; ATP for adenosine triphosphate; ATPase for adenosine triphosphatase; DPN for diphosphopyridine nucleotide; DPNH for diphosphopyridine nucleotide (reduced form); $P_i$ for inorganic phosphate.

TABLE I

CHEMICAL COMPOSITION OF INSECT FLIGHT MUSCLE

| Compound[a] | Tissue Component | | | Species | Reference |
|---|---|---|---|---|---|
| | Whole Muscle | Cytoplasm | Mitochondria | | |
| Protein[a] | 168 | 48 | 120 | *Locusta migratoria* (Linnaeus) | 120 |
| Total P[b] | 17 | 12 | 5 | *Musca domestica* Linnaeus | 83 |
| Acid soluble P[c] | 57 | 85 | 17 | *M. domestica* | 83 |
| Lipid P[c] | 26 | 10 | 64 | *M. domestica* | 83 |
| Nucleic acid P[c] | 9 | 3 | 6 | *M. domestica* | 83 |
| Protein P[c] | 8 | 2 | 13 | *M. domestica* | 83 |
| $Na^{+}$[d] | 16 | — | — | *L. migratoria* | 120 |
| $K^{+}$[d] | 99 | — | — | *L. migratoria* | 120 |
| $Ca^{++}$[d] | 0.7 | — | — | *L. migratoria* | 120 |
| $Mg^{++}$[d] | 14 | — | — | *L. migratoria* | 120 |
| Glycogen[e] | 4 | — | — | *L. migratoria* | 6 |
| Glycogen[e] | 32 | — | — | *M. domestica* | 83 |
| Glycogen[g] | 33 | — | — | *M. domestica* | 110 |
| Glucose[f] | 4 | — | — | *L. migratoria* | 6 |
| Glucose[g] | 7 | — | — | *M. domestica* | 110 |
| Trehalose[g] | 28 | — | — | *M. domestica* | 110 |
| Trehalose[f] | 21 | — | — | *L. migratoria* | 6 |
| Glutamine[f] | 9 | — | — | *Schistocerca gregaria* (Forskål) | 4 |
| Glutamine[h] | 28 | — | — | *M. domestica* | 110 |
| Glutamate[f] | 1 | — | — | *S. gregaria* | 4 |
| Glutamate[h] | 25 | — | — | *M. domestica* | 110 |
| Proline[h] | 16 | — | — | *M. domestica* | 110 |
| ATP[f] | 8.1 | 8.06 | 0.02 | *Calliphora erythrocephala* (Meigen) | 74 |
| ADP[f] | 4.8 | 4.7 | 0.01 | *C. erythrocephala* | 74 |
| AMP[f] | 1.5 | 1.3 | 0.2 | *C. erythrocephala* | 74 |
| Arginine-phosphate[f] | 0.7 | — | — | *Apis mellifera* Linnaeus | 68 |
| Glucose-6-phosphate[f] | 0.10 | — | — | *L. migratoria* | 6 |
| Glucose-6-phosphate[i] | 8 | — | — | *M. domestica* | 110 |
| Glucose-6-phosphate[f] | — | 6 | — | *C. erythrocephala* | 74 |
| Fructose-1,6-diphosphate[f] | 0.25 | — | — | *L. migratoria* | 6 |
| Dihydroxyacetone phosphate[f] | 0.15 | — | — | *L. migratoria* | 6 |
| α-Glycerophosphate[f] | 0.45 | — | — | *L. migratoria* | 6 |
| α-Glycerophosphate[i] | 21 | — | — | *M. domestica* | 111 |
| Pyruvate[f] | 0.2 | — | — | *L. migratoria* | 6 |
| Lactate[f] | 0.35 | — | — | *L. migratoria* | 6 |
| α-Ketoglutarate[f] | 0.1 | — | — | *L. migratoria* | 6 |
| Malate[f] | 0.3 | — | — | *L. migratoria* | 6 |
| Citrate[f] | 0.9 | — | — | *L. migratoria* | 6 |
| Carnitine[f] | 5 | — | 0 | *M. domestica* | 33 |

[a] = mg./gm. wet weight; [b] = μg./thorax; [c] = per cent total $P_i$ in tissue component; [d] = micro-atoms/gm. wet weight; [e] = μM glucose equivalents/gm. wet weight; [f] = μM/gm. wet weight; [g] = per cent total soluble $C^{14}$, 120 min. after $C^{14}$-glucose injection; [h] = per cent total soluble $C^{14}$, 30 min. after $C^{14}$-acetate injection; [i] = per cent total acid soluble $P^{32}$.

serves during flight [Wigglesworth (108); Williams, Barnes & Sawyer (109)] and its rapid catabolism by flight muscle [Sacktor (82)], indicate that glycogen provides a major vehicle for storage of flight energy, which can be quickly mobilized to meet the metabolic requirements of the muscle. Although these data clearly point to the importance of glycogen, recent findings show that other carbohydrates, particularly trehalose, could provide readily available sources of energy. Trehalose was identified as the principal blood sugar in several insect species by Wyatt & Kalf (117); its concentration in the blow fly, *Phormia*, can be as high as 3000 mg. per cent [Evans & Dethier (30)]. In the flight muscle of *Locusta*, Bücher & Klingenberg (6) found trehalose to be present in a concentration of 21 $\mu$M/gm. wet weight, an amount severalfold that of glycogen. Furthermore, we know that the disaccharide was metabolized by *Musca* flight muscle at rates comparable to those of glycogen and glucose [Sacktor (82)], that thoracic muscle of the woodroach, *Leucophaea*, has a very active trehalase [Zebe & McShan (122)], that trehalose supported flight in *Drosophila* [Wigglesworth (108)], and that its concentration in blood and in flight muscle diminished markedly during the continous flight of *Phormia* [Evans & Dethier (30)] and of *Locusta* [Bücher & Klingenberg (6)]. Despite this information, we have little data concerning the comparative significance of trehalose and glycogen as energy reservoirs for flight or, even more revealing, on the sequence in which these carbohydrates are mobilized during flight. On this point, Williams, Barnes & Sawyer (109) showed that, although glycogen diminished throughout the flight of flies, less was used in the earlier portions of the flight than in the later phases. This suggests a possible preferential consumption of trehalose in the earlier periods of activity [see Sacktor (87)].

Special attention should be given to the possible contribution of amino acids as energy-furnishing reserves for flight. Free amino acids represent from 50 to 85 per cent of the non-protein nitrogen in insect blood, and their concentration in insects is about fifty times that found in human serum [Buck (8)]. In house flies, particularly high levels of proline, glutamate, and glutamine were reported by Winteringham (110) 30 minutes after $C^{14}$-acetate injection. In the thoracic muscle of the locust, *Schistocerca*, the glutamate plus glutamine concentration is almost 10 $\mu$M/gm. wet weight [Bellamy (4)], the glutamine to glutamate ratio being 11:1. Perhaps more than by coincidence, these key intermediates were found to be rapidly oxidized with concomitant synthesis of ATP by flight muscle preparations from *Musca* [Sacktor (82); Sacktor & Cochran (93)] and from the orthopterans, *Locusta* [Rees (76)] and *Periplaneta* [Fukami & Tomizawa (36)]. On the other hand, there is no evidence for the liberation of free ammonia in flight [Chadwick (9)]. This apparent contradiction may be adequately explained, however, by speculating that in flight muscle, for each mole of amino acid oxidized, one mole of glutamate will be amidated to glutamine. Similar dismutations are well-known in mammalian tissues, as well as in several insect tissues [Kilby &

Neville (51)], but their quantitative and qualitative demonstrations in flight muscle are needed.

The energetics of flight muscle are intimately interwoven with the metabolism of phosphorylated intermediates. Therefore, it is not surprising to find that flight muscle of *Musca* contains approximately half the total P of the fly [Sacktor (83)]. As shown in Table I, 50 per cent of the P from muscle is in the acid-soluble fraction and 25 per cent is incorporated as phospholipid. In the mitochondria of the muscle, however, almost two-thirds of the P is found as lipid-P. Unfortunately, there has been little characterization of this important component of insect mitochondria. Such studies are particularly needed now in view of recent findings by Wojtczak & Wojtczak (115) of a mitochondrial lipid that in minute quantities strongly inhibits oxidative phosphorylation. (This observation will be discussed in greater detail later in this review.) In contrast to mitochondria, the soluble cytoplasm (sarcoplasm) of flight muscle contains comparatively little lipid-P; 85 per cent of its P is acid-soluble [Sacktor (83)].

The quantities and distribution of several of the more important acid-soluble phosphorylated intermediates are included in Table I. Flight muscle contains all the Meyerhof-Embden glycolytic intermediates [Bücher & Klingenberg (6); Winteringham, Bridges, & Hellyer (112)]. Sacktor (83) reported the presence in *Musca* flight muscle of components of the pentose-shunt pathway. This supplements the enzymatic demonstration by Chefurka (22) of this pathway in the same species. One might assume that, since the enzymes concerned with both hexose degradation systems are found in the soluble cytoplasm [Chefurka (24); Sacktor (82)], their phosphorylated metabolites would be found exclusively in the extramitochondrial portion of the muscle; however, this is not the case. Sacktor (83) found substantial amounts of glucose-6-phosphate, assayed enzymatically by reduction of triphosphopyridine nucleotide in presence of glucose-6-phosphate dehydrogenase, in mitochondria as well as in cytoplasm. Ketopentoses, such as ribulose- or xylose-phosphate, determined by their characteristic absorption spectra of their orcinol reaction products, were also demonstrated by Sacktor (83) in *Musca* flight muscle mitochondria. A satisfactory explanation for this apparent enigma has yet to be developed. Of particular significance is the unusually high concentration of $\alpha$-glycerophosphate in insect flight muscle. Winteringham (111) reported that $\alpha$-glycerophosphate represents 20 per cent of the total acid-soluble P of *Musca* flight muscle. Special importance has been attributed to $\alpha$-glycerophosphate [see Sacktor (85)], and the enzymatic reactions related to its formation and subsequent oxidation will be considered separately.

Sacktor & Cochran (90) estimated that approximately 90 per cent of the nucleotide fraction of house fly flight muscle contains adenine. The adenine nucleotides, ATP, ADP, and AMP, were assayed in *Phormia* by Levenbook (62), in *Calliphora* by Price & Lewis (74), and in *Apis* by Maruyama & Moriwaki (68). Comparisons of the concentrations of these nucleotides, as

well as of P, in flight muscle mitochondria, with those reported for mammalian mitochondria reveal the remarkable fact that the levels in insect mitochondria are only 20 per cent those in mammalian mitochondria, despite the tremendous respiratory activity of insect flight muscle. Price & Lewis (74) found that the bulk of the nucleotides of *Calliphora* flight muscle is localized in the cytoplasm; less than 3 per cent are in mitochondria. On the other hand, Levenbook (62) claimed that 60 to 70 per cent of the adenine nucleotides of *Phormia* are in mitochondria; however, these data were not obtained by actual analysis but deduced from unspecific optical density measurements at 260 mμ. Sacktor (83) found that the distribution of the adenine nucleotides in *Musca* agrees with the values reported by Price & Lewis (74).

Another striking difference between the phosphorylated intermediates of insect flight muscle and that of skeletal muscle from other animals is in the concentration of phosphagen. For example, Meyerhof (70) found that, in crustacean muscle, as much as 75 per cent of the total acid soluble P is arginine-phosphate, and LePage (59) reported over 16 μM creatine-phosphate/gm. wet weight of skeletal muscle of rat. On the other hand, Maruyama & Moriwaki (68) estimated that the honey bee thorax contained only 0.7 μM arginine-phosphate/gm. wet weight. This relatively low arginine-phosphate content, as well as other findings, which will be discussed below, suggests to me that the entire question of the nature and role of the phosphagen in insect flight muscle needs re-examination. Schutze (98) and Baldwin & Needham (2) were the first to report arginine-phosphate in insects. Baldwin & Needham (2) detected arginine by the Sakaguchi color test and the arginase-urease method after hydrolysis of the phosphagen-containing extracts of thoraces from *Calliphora*. These authors noted, however, that, although their evidence pointed to the occurrence of arginine-phosphate in fly muscle, its presence was not ascertained, since sufficient amounts were never obtained to permit preparation and analysis of the pure compound nor was a crystalline derivative of arginine prepared and characterized. Nevertheless, most investigators cite Baldwin & Needham's paper and rely primarily on evidence obtained from rates of hydrolysis in claiming the presence of arginine-phosphate and in assuming its role as the phosphagen in insects [Levenbook (61); Schutze (98); Winteringham, Bridges & Hellyer (112)]. It should be emphasized that the Lohmann reaction:

$$\text{ATP} + \text{arginine} \rightleftarrows \text{ADP} + \text{arginine-phosphate} \qquad 1.$$

has never been demonstrated in flight muscle nor, to my knowledge, in any other insect tissue. Moreover, efforts to synthesize arginine-phosphate with *Musca* mitochondria that actively generated ATP during oxidative phosphorylation were unsuccessful [Sacktor (81)]. More recently, Tomizawa & Fukami (105) were unable to find any trace of $P^{32}$ incorporation into arginine-phosphate in similar experiments using muscle homogenates of *Locusta*.

In recent years, the general assumption that the creatine-phosphate char-

acteristic of vertebrate muscle is always replaced by arginine-phoshate in the muscle of invertetrates has been modified considerably. Roche and his coworkers have isolated several phosphorylated guanidine derivatives, other than arginine, from annelids and have shown that the worms contain phosphokinases that will phosphorylate these guanido compounds at the expense of ATP [see Thoai & Roche (104)]. In view of the misgivings about the place of arginine-phosphate in insect flight muscle metabolism, the possible occurrence of other labile P compounds in this tissue must be considered. In fact, Sacktor (83) presented evidence consistent with this possibility. In the barium-soluble alcohol-insoluble fraction of trichloroacetic acid extracts of *Phormia* or *Musca* thoraces, a compound was found that was appreciably more labile than was arginine-phosphate. In Table II, this unknown is compared with arginine-phosphate that was prepared from lobster tail muscle according to the procedure of Meyerhof & Lohmann (71). With arginine-phosphate, little apparent inorganic phosphate was observed when $P_i$ was assayed by the methods of Fiske & Subbarow (32) or Sumner (103). P was completely hydrolyzed in 1*N* acid, boiled for 7 min., and stoichiometric agreement was found between the liberated $P_i$ and arginine. In contrast, the labile compound from flies was hydrolyzed by the methods used for apparent inorganic phosphate, and the arginine content did not account for the $P_i$ liberated. Paper chromatography of the hydrolyzed compound from thoraces revealed two ninhydrin positive spots, only the smaller one of which reacted as arginine. The identification of this unknown is continuing, and, as yet, nothing can be said of its metabolic function. Nevertheless, these data focus additional attention on a serious gap in our knowledge of the nature and role of the phosphagen in the physiology of insect flight muscle.

Another problem still to be resolved is to determine whether or how carni-

TABLE II

PHOSPHAGEN OF INSECT FLIGHT MUSCLE

| Preparation | Phosphate Assay | | Arginine Assay |
|---|---|---|---|
| | Method | μM | |
| Lobster tail muscle | | | |
| Arginine-phosphate | Fiske-Subbarow | 0.06* | 0.01* |
| | Sumner | 0.08* | — |
| | 7 min. 1*N* HCl | 1.03* | 1.01* |
| House fly thorax | | | |
| Ba-soluble, alcohol-insoluble fraction | Fiske-Subbarow | 0.48† | 0.00† |
| | Sumner | 1.27† | — |
| | 7 min. 1*N* HCl | 2.10† | 0.20† |

* μM/sample.

† μM/100 thoraces.

tine functions in insect flight muscle. Fraenkel (33) reported that *Musca* flight muscle possesses a relatively high carnitine content, 2200 μg./gm. of dry muscle (Table I). The vitamin is located exclusively in the extramitochondrial portion of the muscle. Despite the abundance of carnitine throughout the animal kingdom, very little is known about its role in metabolism. An important step in this direction was made by Friedman & Fraenkel (35), who showed that acetylcarnitine can acetylate coenzyme A, and they postulated that carnitine may play a role in such group transfer reactions. Further studies in this direction are obviously needed.

### Respiratory Enzymes of Flight Muscle Mitochondria

The rapid rates of muscular contraction and energy expenditure during flight are reflected in the enormous respiratory exchange of the active insect. Keilin's (49) historic observations on the oxidation and reduction of cytochrome in flight muscle gave the first indication of the participation of cytochromes in respiratory mechanisms. Several decades later, Watanabe & Williams (106), in an admirable study, demonstrated the presence of high titers of cytochromes in the isolated sarcosomes, or mitochondria, of flight muscle. Since then the spectra and kinetics of the respiratory enzymes in insect mitochondria have been studied in greater detail, and we now have a better appreciation of the components of the respiratory chain in flight muscle, the relative concentrations of these components, and the effects of various substrates and inhibitors on their steady states and kinetics.

In the paper that described the rediscovery of cytochrome, Keilin (49) noted that, among all organisms examined, the highest concentration of cytochrome is found in thoracic muscle of flying insects and that this muscle is the best material for the study of the absorption spectra of these pigments. Using a microspectroscopic ocular, Keilin inspected 40 species of insects representing most of the major orders and saw four absorption bands corresponding to what are now known as the $\alpha$ bands of cytochromes-*a*, -*b*, and -*c* and the combined $\beta$ bands of cytochromes-*b* and -*c*. Using similar techniques, Williams and co-workers (63, 72, 97, 99, 106) and Sacktor (78) also detected these bands in insect muscle or in the mitochondria thereof. Chance (12, 13) developed rapid and sensitive spectrophotometric techniques which measured quantitative changes in the optical density of these pigments in blow fly mitochondria. A more detailed study of the respiratory components of *Musca* flight muscle mitochondria was made by Chance & Sacktor (17) and Estabrook & Sacktor (28). When mitochondria were reduced enzymically with $\alpha$-glycerophosphate, succinate, or DPNH, we observed, at room temperature, spectral peaks at 605, 563, and 550 mμ, representing the $\alpha$ bands of reduced cytochromes-*a*, -*b*, and -*c*, respectively. The absorption bands found at about 520 to 530 mμ represent a mixture of $\beta$ bands of the reduced cytochromes. The trough of the flavoprotein was distinctly seen at 470 mμ, while the peak of cytochrome-$a_3$ was at 445 mμ. The Soret bands of cytochromes-*b* and -*c*, at 430 and 419 mμ respectively, were distinguished as shoulders on the

spectrophotometric trace. In the ultraviolet region, the absorption increased slowly toward 320 mμ without any peak at 340 mμ of reduced pyridine nucleotide. When the oxidized pigments were reduced non-enzymically with sodium dithionite, a similar spectrum, differing only in a greater absorption at about 562 mμ, was observed. Klingenberg & Bücher (52) recently reported similar spectral curves with flight muscle mitochondria from *Locusta*.

In other spectroscopic experiments, Chance & Sacktor (17) found that antimycin-A treatment of the aerobic mitochondria in the presence of substrate caused the appearance of the characteristic absorption bands of cytochrome-*b* at 430 and 560 mμ. Preliminary studies were made of the effect of adding antimycin-A to an anaerobic suspension of mitochondria. This led to a rather complicated intensification of the absorption bands of cytochrome-*b* and of a "*c*" component. The addition of nitrite or formate to the aerobic mitochondria caused a spectrophotometric change which is incompletely understood at this time. Chance & Sacktor (17) reported that either one of these substances caused the appearance of a large absorption band with a peak at 426 mμ, perhaps a peroxidase complex [Chance (14)].

These difference spectra measured at room temperature resemble those described for mammalian mitochondria. However, when insect flight muscle mitochondria were examined at the temperature of liquid nitrogen, which brings about a marked sharpening and intensification of the absorption bands of cytochromes, notable differences in cytochrome content from that observed with their mammalian counterpart were revealed. Estabrook & Sacktor (28) found that *Musca* flight muscle mitochondria, reduced enzymically with α-glycerophosphate, succinate, or DPNH and examined at −190°C., showed the expected absorption bands at 598, 561, 548, and 545 mμ indicative of cytochromes-*a*, -*b*, -$c\alpha_1$, and -$c\alpha_2$. Unexpected was the complete absence of an absorption band of cytochrome-$c_1$ at 554 mμ. It was evident from these experiments that flight muscle mitochondria had a rather high concentration of cytochrome-*c*. The possibility that this high titer was sufficient to obscure the band of reduced cytochrome-$c_1$, if, indeed, the latter was present, was eliminated by removing cytochrome-*c* from the preparation. Again, no evidence of cytochrome-$c_1$ at 554 mμ was seen; instead, another pigment with an α band maximum at 551 mμ was observed. In contrast with the enzymic reduction of the cytochromes, reduction with dithionite revealed a pigment at 555 mμ in addition to the cytochrome with an absorption maximum at 551 mμ. In other experiments, the enzymic reduction of the pigments was carried out in the presence of antimycin-A. Besides the reduction of cytochrome-*b*, a second pigment with an absorption maximum at 555 mμ appeared.

These and other spectral studies with insect flight muscle mitochondria, coupled with an evaluation of the enzymic activities of these preparations, permitted Estabrook & Sacktor (28) to postulate a respiratory chain for insect mitochondria. The scheme is shown in Figure 1.

The pigment that had an α absorption band at 551 mμ at the tempera-

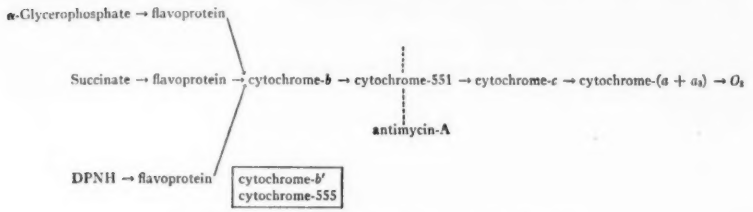
α-Glycerophosphate → flavoprotein
Succinate → flavoprotein → cytochrome-b → cytochrome-551 → cytochrome-c → cytochrome-(a + a3) → O2
antimycin-A
DPNH → flavoprotein
cytochrome-b'
cytochrome-555

FIG. 1. Respiratory chain of insect mitochondria.

ture of liquid nitrogen and reducible by substrates and ascorbic acid was designated simply by its wavelength. In a similar manner, the pigment that appeared on the addition of antimycin-A or dithionite but was not reduced in anaerobiosis was termed cytochrome-555. The additional absorption at about 562 mμ observed in the presence of antimycin-A was previously designated cytochrome-$b'$ by Chance (15), who used other mitochondria. These two pigments, cytochromes-555 and -$b'$, were separated from the electron transport chain but placed between substrate and the antimycin-A-sensitive reaction, since no definitive evidence is available as to their function.

In an elegant spectroscopic study of the individual tissues of the Cecropia silkworm, *Platysamia*, Shappirio & Williams (99) reported an absorption band lying at 555 mμ at low temperature in somatic muscle. They identified this band as cytochrome-$c_1$ of mammalian mitochondria, formerly termed "$e$" [Keilin & Hartree (50)]. It should be noted that the 555 mμ band was obtained by reduction with dithionite. Estabrook & Sacktor (28) also found this peak in flight muscle mitochondria when the non-enzymic reducing agent was used or when the suspension was reduced in the presence of antimycin-A. However, Estabrook & Sacktor (28) showed that this pigment in flight muscle was not cytochrome-$c_1$, since previous assessment of the other properties of $c_1$ indicated that it is reduced enzymically by succinate or DPNH or non-enzymically by ascorbic acid, as well as by dithionite, but that it is not reduced by substrates in the presence of antimycin-A. The pigment appearing at 555 mμ does not possess these properties. Instead, such properties characterize cytochrome-551, which is visible in flight muscle after removal of cytochrome-$c$. As shown in Figure 1, the pigment at 555 mμ was separated from the main electron transport chain pending further evidence of its functional position. The presence of cytochrome-551 in somatic muscle or tissues other than the active flight muscle has yet to be observed.

A new component of the respiratory chain has been described recently. This component is called coenzyme Q or ubiquinone, and it is a derivative of 2,3-dimethoxy-5-methylbenzoquinone substituted at position-6 with a polyisoprenoid side chain varying from six to 10 units [Lester & Crane (60)]. Coenzyme Q has wide distribution in animals and plants. It has been demonstrated in preparations from the entire house fly, *Musca*, and the cabbage

butterfly, *Pieris*, by Lester & Crane (60), although it is either not present or of doubtful presence in other arthropods, such as lobster and shrimp. Of particular interest is the finding of these investigators that insect coenzyme Q contains nine isoprene units, whereas there are 10 units in the coenzyme of mammalian tissues. Chance & Sacktor (18) found coenzyme Q in flight muscle mitochondria from the blow fly, *Callitroga*, and showed its rapid enzymic reduction with α-glycerophosphate as substrate. The position of coenzyme Q in the respiratory chain is still unclear, but evidence from several laboratories suggests that it functions between the dehydrogenases and the cytochrome-*c* components.

Chance & Sacktor (17) determined the concentrations of the different respiratory components in *Musca* flight muscle mitochondria by measuring optical density changes, at appropriate wavelengths, caused by the transition from aerobiosis to anaerobiosis upon addition of α-glycerophosphate. These data, summarized in Table III, demonstrate roughly equal concentra-

TABLE III

RELATIVE CONCENTRATIONS AND STEADY STATE REDUCTION OF THE COMPONENTS OF THE RESPIRATORY CHAIN

| Component | Wavelengths* | | Relative concentration | Reduction in steady state |
|---|---|---|---|---|
| | Measurement | Reference | | |
| Cytochrome-$a_3$ | 445 | 510 | 1.4 | 0 to 12% |
| Cytochrome-*a* | 605 | 630 | 1.0 | 2 to 25% |
| Cytochrome-*c* | 551 | 540 | 1.7 | 40% |
| Cytochrome-*b* | 564 | 575 | 0.5 | 38% |
| Flavoprotein | 465 | 510 | 2.2 | 66% |

* Expressed in millimicrons.

tion of the cytochromes. Essentially similar studies by Klingenberg & Bücher (52), using mitochondria from locust flight muscle, confirmed these results. The concentrations of locust pigments were 0.95, 1.45, 1.0, and 0.88 for cytochromes-*b*, -*c*, -*a*, and -$a_3$, respectively. The higher cytochrome-*b* value estimated by Klingenberg & Bücher was in part attributable to the inclusion in their calculations of the additional absorption caused by antimycin-A in the cytochrome-*b* region. The relative concentrations of the cytochromes in the respiratory chain of insect flight muscle mitochondria resemble those of mammalian heart muscle mitochondria. However, Chance & Sacktor (17) pointed out that insect mitochondria differ strikingly from mammalian mitochondria in their ratio of pyridine nucleotide content to cytochrome. For instance, liver mitochondria contain up to 40:1 pyridine nucleotide to cytochrome-*a*, whereas this ratio in *Musca* is very low. In *Locusta* mitochondria, Klingenberg & Bücher (52) found that the ratio of the

two components was 4:1. It should be noted that the amount of pyridine nucleotide per gm. mitochondrial protein does not differ appreciably between flight muscle and mammalian preparations [Klingenberg, Slenczka & Ritt (53); Sacktor (83)]. Instead, the difference between the pyridine nucleotide-cytochrome relationship results from a greater concentration of cytochrome in flight muscle. Of particular interest, although its significance is not clear, is the claim of Klingenberg, Slenczka & Ritt (53) that triphosphospyridine nucleotide was absent from *Locusta* flight muscle mitochondria,[3] in spite of the fact that the isocitrate dehydrogenase of honey bee flight muscle mitochondria is specific for TPN [Hoskins, Cheldelin & Newburgh (43)]. The coenzyme Q concentration in mitochondria of *Callitroga* flight muscle was approximately sevenfold that of cytochrome-*c* [Chance & Sacktor (18)], a value in agreement with those found in mammalian mitochondria.

The steady state percentage reduction of each respiratory component was calculated by Chance & Sacktor (17). The ratio of intensity of the steady-state reduced-absorption band to the total effect caused by the transition from aerobiosis to anaerobiosis upon addition of $\alpha$-glycerophosphate was measured. In the case of cytochromes-*a* and -$a_3$ (Table III), a range of values is given, since the extent of reduction of these components increased as the respiration proceeded in the first minute or so of respiration. The smaller values were, however, representative of the initial activity. The percentage reductions of cytochromes-*b* and -*c* were about equal, while that of flavoprotein was somewhat greater. This is in accordance with the general effect noted in most respiratory chains: the extent of reduction decreases as one progresses from the dehydrogenase to the oxidase. Chance & Sacktor (17), with mitochondria from *Musca*, observed a considerable increase in absorption at 340 m$\mu$ with respect to 374 m$\mu$ upon addition of $\alpha$-glycerophosphate. A steady state value of 40 per cent reduction was computed. Values as high as 72 per cent were found in *Locusta* by Klingenberg, Slenczka & Ritt (53), who attributed these optical density changes to DPN reduction. Since, as shown in Figure 1, DPN is not on the direct pathway of $\alpha$-glycerophosphate oxidation by mitochondria, Klingenberg, Slenczka & Ritt (53) interpreted this reduction of DPN as a reversal of oxidative phosphorylation.

The substrate is of great importance for measurements of respiratory activity of flight muscle mitochondria. Our early experiments revealed that $\alpha$-glycerophosphate is the substrate for which mitochondria had the highest activity [Chance & Sacktor (17); Chance & Williams (19); Sacktor (84); Sacktor & Cochran (89); Zebe, Delbruch & Bücher (120)]. As shown in Table IV, the rate of $\alpha$-glycerophosphate oxidation was tenfold that of succinate; occasional values of 20 were noted. $\alpha$-Ketoglutarate showed about the same activity as succinate, but isocitrate, pyruvate, and malate were considerably lower.

[3] Professor Bücher has recently told me that small quantities of his coenzyme have now been found in *Locusta* mitochondria.

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The steady state percentage reduction of each respiratory component was calculated by Chance & Sacktor (17). The ratio of intensity of the steady-state reduced-absorption band to the total effect caused by the transition from aerobiosis to anaerobiosis upon addition of $\alpha$-glycerophosphate was measured. In the case of cytochromes-*a* and -$a_3$ (Table III), a range of values is given, since the extent of reduction of these components increased as the respiration proceeded in the first minute or so of respiration. The smaller values were, however, representative of the initial activity. The percentage reductions of cytochromes-*b* and -*c* were about equal, while that of flavoprotein was somewhat greater. This is in accordance with the general effect noted in most respiratory chains: the extent of reduction decreases as one progresses from the dehydrogenase to the oxidase. Chance & Sacktor (17), with mitochondria from *Musca*, observed a considerable increase in absorption at 340 m$\mu$ with respect to 374 m$\mu$ upon addition of $\alpha$-glycerophosphate. A steady state value of 40 per cent reduction was computed. Values as high as 72 per cent were found in *Locusta* by Klingenberg, Slenczka & Ritt (53), who attributed these optical density changes to DPN reduction. Since, as shown in Figure 1, DPN is not on the direct pathway of $\alpha$-glycerophosphate oxidation by mitochondria, Klingenberg, Slenczka & Ritt (53) interpreted this reduction of DPN as a reversal of oxidative phosphorylation.

The substrate is of great importance for measurements of respiratory activity of flight muscle mitochondria. Our early experiments revealed that $\alpha$-glycerophosphate is the substrate for which mitochondria had the highest activity [Chance & Sacktor (17); Chance & Williams (19); Sacktor (84); Sacktor & Cochran (89); Zebe, Delbruch & Bücher (120)]. As shown in Table IV, the rate of $\alpha$-glycerophosphate oxidation was tenfold that of succinate; occasional values of 20 were noted. $\alpha$-Ketoglutarate showed about the same activity as succinate, but isocitrate, pyruvate, and malate were considerably lower.

[3] Professor Bücher has recently told me that small quantities of his coenzyme have now been found in *Locusta* mitochondria.

TABLE IV

MITOCHONDRIAL RESPIRATION

| Substrate | Relative activity |
|---|---|
| α-Glycerophosphate | 100 |
| Succinate | 10 |
| α-Ketoglutarate | 9 |
| Isocitrate | 2 |
| Pyruvate | 2 |
| Malate | 1 |

The high activity of isolated flight muscle mitochondria toward α-glycerophosphate is notably influenced by several experimental variants. Of particular significance for these measurements is the extreme lability of the α-glycerophosphate oxidase. The adverse effects of prolonged incubation and shaking associated with the usual manometric techniques of respirometry were clearly demonstrated by Sacktor & Cochran (93) and Chance & Sacktor (17). Also of great importance for maximal α-glycerophosphate oxidase activity are the age of the fly from which the mitochondria were prepared and the concentrations of substrate and mitochondria [Chance & Sacktor (17); Estabrook & Sacktor (29); Sacktor (82)]. Differences in the earlier manometric measurements of respiratory activity with α-glycerophosphate and the more recent experiments that advantageously measured respiration with an oxygen electrode are thus understandable, and a full recognition of these effects has not been appreciated in some instances. These observations led Chance & Sacktor (17) to suggest that there is present in mitochondria a cofactor for α-glycerophosphate oxidase activity that is expended or, perhaps, made unavailable during the course of the oxidation reaction.

Chance & Sacktor (17) determined that flavoprotein and cytochrome accepted electrons at a rate consistent with the extraordinary respiratory activity obtainable with α-glycerophosphate. In these experiments, electron transfer to oxygen was blocked by prior addition of azide, and the reactions were carried out at 6°C. so that the rates of reduction were slow enough to be measured conveniently. Addition of α-glycerophosphate caused a complete reduction of the components, the reactions following roughly exponential courses. On the basis of half-times, the reduction of cytochromes-*b* and -*c* and flavoprotein proceeded at about the same rate, the half-times being about 1 sec. The rates of change of concentration, calculated from the initial slopes, were about equal for cytochrome-*c* and for flavoprotein calculated on an iron basis, while the initial rate for cytochrome-*b* appeared to be slower in this particular preparation. The respiratory rate at this low temperature was 0.43 μM Fe/sec., and this was adequately accounted for in terms of the rate of reduction of cytochrome-*c* and flavoprotein.

In view of the high activity of the α-glycerophosphate oxidase system of flight muscle mitochondria, Chance & Sacktor (17) made calculations to determine whether the enzyme had sufficient activity to account for the respiratory rate of the insect in flight. In these *in vitro* studies, the highest value of $K_{4c}$ ($\mu$M $O_2$/sec./$\Delta D_{550}$) was 620 at 25°C., and turnover numbers of cytochrome-*c* gave values of 34 to 68 sec.$^{-1}$. Knowing the respiratory rate of the fly in flight from Chadwick (10) and the concentration of cytochrome-*c* in *Musca* thorax, the turnover number of cytochrome-*c* in the flying insect was estimated to be 50 sec.$^{-1}$. This *in vivo* value compared with that observed in the isolated mitochondria. Thus, the respiratory rate of isolated mitochondria in the presence of α-glycerophosphate and dibromophenol or ADP largely accounts for the respiratory rate of the flying fly [Chance & Sacktor (17); Sacktor (86, 87)]. The lesser activities with other substrates, for example, the Krebs cycle acids, are noteworthy. At most, the rates of oxidation with these intermediates account for the respiration of the fly at rest. Our results with *Musca* flight muscle mitochondria are in sharp contrast with those of Zebe, Delbruck & Bücher (120), who reported that the respiratory capacity of *Locusta* preparations (oxidizing α-glycerophosphate), while high in comparison with other substrates and with the resting respiration of the flight muscle, was not sufficient to explain the working respiration of the insect.

### Oxidative Phosphorylation in Insect Mitochondria

Oxidative phosphorylation is a vital process in the economy of aerobic cells, probably accounting for over 90 per cent of all the ATP generated from ADP and $P_i$ at the expense of the energy liberated during catabolism of foodstuffs. Both electron transport and oxidative phosphorylation take place in mitochondria. During the exergonic passage of electrons from substrate to molecular oxygen, via pyridine nucleotide, flavoproteins, and cytochromes, ATP is generated in coupled reactions at specific points in the respiratory chain. Despite the great importance of this process, the enzymatic details of oxidative phosphorylation are still essentially unknown. For the current status of our information in this area, the reader is referred to the reviews of Chance & Williams (20) and Lehninger *et al.* (58). Oxidative phosphorylation in insects was first demonstrated by Sacktor (81), who used flight muscle mitochondria of *Musca*. This was soon followed by the independent efforts of Lewis & Slater (65) with mitochondria from the blow fly, *Calliphora*, and later confirmed and extended by others, including Gonda, Traub & Avi-Dor (41) with *Aedes;* Ito & Horie (47) with *Bombyx;* Cochran & King (26) with *Periplaneta;* Rees (76), Tomizawa & Fukami (105), and Klingenberg & Bücher (52) with *Locusta;* and Wojtczak & Wojtczak (114) with *Galleria*.

A useful guide in determining how much ATP is synthesized by intracellular respiration, as well as in identifying those steps in the respiratory chain that are associated with phosphorylation, has been to measure the yield of oxidative phosphorylation obtained in the oxidation of different substrates.

These yields are often expressed as P:O ratios, the number of atoms of phosphorus esterified per atom of oxygen consumed. P:O values from 2 to 3 have been obtained with substrates, such as glutamate, α-ketoglutarate, and pyruvate, the oxidations of which proceed via DPN [Cochran & King (26); Gregg, Heisler & Remmert (42); Rees (76); Sacktor & Cochran (89, 93)]. On the other hand, according to Figure 1, the mitochondrial oxidation of α-glycerophosphate and succinate does not directly involve pyridine nucleotide. With these substrates, ratios approaching 2 have been observed [Sacktor & Cochran (93)]. These results with insect mitochondria are consistent with the viewpoints summarized by Lehninger *et al.* (58) and Chance & Williams (20) for mammalian preparations; during the passage of a pair of electrons from substrate to molecular oxygen via pyridine nucleotide, three moles of ATP are generated, whereas from substrates, such as succinate or, as we have shown, α-glycerophosphate, two moles of ATP are formed

The necessary conditions for demonstrating oxidative phosphorylation in insect mitochondria were described by Sacktor (81). Of particular importance was the observation that serum albumin stimulated phosphorylation. In fact, *Musca* flight muscle mitochondria were prepared that showed an absolute requirement for serum albumin and no phosphorylation could be demonstrated in the absence of the protein. This effect of albumin has since been confirmed and extended by Lewis & Slater (65), Slater & Lewis (101), and Wojtczak & Wojtczak (115). Although the need for serum albumin in phosphorylation is best seen in insect mitochondria, parallel effects have recently been found in mammalian preparations [Polis & Shmukler (73); Pullman & Racker (75); Stern & Timonen (102)]. Sacktor, O'Neill & Cochran (95) showed that iodination of albumin or reacting the protein with *p*-chloromercuribenzoate inactivated the albumin. Dialysis of this *p*-chloromercuribenzoate-inactivated albumin against cysteine to remove the combined inhibitor restored the original activity to the albumin. These studies suggest that the free sulfhydryl group of albumin is necessary for the action of the protein on phosphorylation.

Pullman & Racker (75), in experiments on the reactivation of oxidative phosphorylation with aged mammalian liver mitochondria, and Sacktor, O'Neill & Cochran (95), using freshly prepared flight muscle mitochondria, showed the specificity of albumin. Other proteins were either inactive or, like β-lactoglobulin, had a small effect. In the light of these experiments, it appeared unlikely that serum albumin acted only by providing a satisfactory osmolar environment which preserved the structural integrity of the mitochondria [Sacktor (81); Watanabe & Williams (107)]. The first clue to a possible explanation for the serum albumin effect came from Pullman & Racker (75), who showed that albumin combined with an uncoupler of oxidative phosphorylation. This uncoupler, which was released from the mitochondria, was tentatively identified with mitochrome, an electrophoretically homogeneous heme protein, isolated at that time from liver mitochondria, which inhibited aerobic phosphorylation and which was counteracted by serum al-

bumin [Polis & Shmukler (73)]. Later, however, Hulsmann, Elliott & Rudney (44) showed that the active component of mitochrome was not the heme protein, but a lipid adsorbed on the protein, and was extractable with organic solvents. More recently, this lipid material was found to contain fatty acids, among which oleic, palmitic, stearic, linoleic, and linolenic have been identified [Hulsmann, Elliott & Slater (45); Wojtczak & Wojtczak (115)]. This lipid uncoupler is most likely identical with the newly described "U" factor of Lehninger & Remmert (57). Presumably, the ability of serum albumin to prevent uncoupling stems from its capacity to bind fatty acids. Since the free sulfhydryl group was necessary for the action of albumin on phosphorylation, this perhaps represents a possible site of interaction between serum albumin and the inhibitor.

The experiments of Hulsmann, Elliott & Rudney (44) and Lehninger & Remmert (57), using mammalian liver mitochondria, and those of Wojtczak & Wojtczak (114) and Avi-Dor & Gonda (1), using wax-moth, mosquito, and house-fly mitochondria, revealed that the inhibitor of phosphorylation also inhibited the ATP-phosphate exchange reaction, had no effect on the ATP-ADP exchange, and stimulated ATPase activity. These enzymic processes are intimately related to aerobic phosphorylation, as shown in the following equations:

$$\text{Carrier} + \text{X} \overset{\text{electron transfer}}{\leftrightharpoons} \text{carrier} \sim \text{X} \qquad 2.$$

$$\text{Carrier} \sim \text{X} + \text{P}_i \rightleftharpoons \text{carrier} + \text{X} \sim \text{P} \qquad 3.$$

$$\text{X} \sim \text{P} + \text{ADP} \rightleftharpoons \text{X} + \text{ATP} \qquad 4.$$

Reactions 3 and 4 are involved in ATP-phosphate exchange, Reaction 4 in ATP-ADP exchange. Reactions 4 and 3 followed by an irreversible hydrolysis of carrier ~X,

$$\text{Carrier} \sim \text{X} \xrightarrow{\text{H}_2\text{O}} \text{carrier} + \text{X}, \qquad 5.$$

are the basis of mitochondrial ATPase activity. The effects of the lipid inhibitor on oxidative phosphorylation, mitochondrial ATPase, and ATP-phosphate exchange can be accounted for by assuming that the inhibitor, or U factor, promoted the irreversible Reaction 5. The lack of an inhibitory effect on ATP-ADP exchange is understandable if one assumes that the rate of Reaction 4 is far greater than that of Reaction 3.

Lehninger & Remmert (57), using liver mitochondria, and Lewis & Fowler (64), using blow fly flight muscle mitochondria, showed that the endogenous lipid uncoupling agent was generated enzymatically. Lehninger & Remmert (57) argued that it is premature to conclude that the formation of this lipid is a non-specific consequence of the disruption of cell or mitochondrial structure and of the subsequent lipolysis to produce free fatty acid and that this mechanism has no particular physiological significance. On the con-

trary, the promotion of mitochondrial swelling by the U factor suggested to these authors that the lipid is an important endogenous or intracellular factor in reversible swelling and contracting of mitochondria, a physiological process currently receiving considerable attention as a mechanism for metabolic regulation by mitochondria.

The relationship between aerobic phosphorylation and ATPase activity is apparent from Reactions 3, 4, and 5. Gilmour & Calaby (39) reported a Mg-activated soluble enzyme, found in extracts of locust muscle, which dephosphorylated ATP. Sacktor (79) found that insect mitochondria had a specific ATPase. This ATPase, from *Musca* flight muscle mitochondria, was activated by Mg and Mn, but not Ca, and cleaved only the terminal phosphate from ATP. Although liberation of phosphate also occurred when ADP was the substrate, it was demonstrated that this activity was caused by the presence of a Mg-activated adenylate kinase, which converted ADP to ATP and AMP and thus provided substrate for ATPase. Confirmatory studies on the mitochondrial ATPase of other insects were made by Gilmour (38), using *Calliphora*, Maruyama (66), using *Vespula*, Sacktor *et al.* (96), using *Periplaneta;* Wojtczak *et al.* (113), using *Galleria*, and Maruyama & Sakagami (69) using *Apis*.

Sacktor (79) found that flight muscle of flies contained three distinct ATP-dephosphorylating enzymes. In addition to the mitochondrial ATPase, an ATPase that was associated with the muscle myofibrils and was Ca-activated was described. This ATPase is the counterpart in insect muscle of the classical ATPase of mammalian muscle. For detailed studies on the physical and enzymic properties of insect actomyosin, the reader is referred to the papers of Gilmour & Calaby (40) and Maruyama (67). The soluble cytoplasm, like the mitochondria, possessed a Mg-activated ATPase, but this could be distinguished from the mitochondrial ATPase by concurrent inorganic pyrophosphatase activity, as well as by differences in the effects of several inhibitors [Sacktor (79)].

Further studies of the nucleotide-dephosphorylating enzymes in the mitochondrial fraction of insect flight muscle, together with the distribution and nature of such enzymes in other fractions, were made by Sacktor & Cochran (90). It was found that *Musca* mitochondria dephosphorylate the triphosphates of adenosine, guanosine, inosine, uridine, and cytidine. The rates of hydrolysis for these nucleotides are in the order listed above. Mitochondria liberate the two labile $P_i$ from the purine nucleoside triphosphates. In contrast, all three $P_i$ are released from the pyrimidine analogues. Mg and Mn activate dephosphorylation, Mn being the more potent of the two. Ca is inhibitory. Dinitrophenol stimulates the hydrolysis of all triphosphonucleotides to the same extent. Adenosine and inosine diphosphates are competitive inhibitors of their respective triphosphatases. ADP is also an extremely effective inhibitor of inosine and guanosine triphosphatases. Inosine diphosphate has no effect on ATPase, but it inhibits guanosine triphosphatase. Neither purine ribodiphosphate inhibits uridine triphosphatase.

These observations provide evidence suggesting that there is in mitochondria a specific nucleoside triphosphatase for each of the nucleoside triphosphates.

In addition to the mitochondria, Sacktor & Cochran (90) showed that other portions of the muscle, myofibrils and cytoplasm, can hydrolyze adenosine, inosine, guanosine, and uridine triphosphates. The enzymes in the cytoplasm are activated by Mg and inhibited by Ca. The dephosphorylation of these four nucleoside triphosphates by the myofibrils is activated by Ca. These findings, plus the fact that these nucleotides also occur in muscle [Bergkvist & Deutsch (5)], suggest that more than one nucleotide may react with the contractile elements *in vivo* and that a re-examination of the physiological role of nucleotides in muscle contraction may be warranted.

As first shown by Lardy & Wellman (56), mammalian mitochondria yield high rates of respiration and maximum P:O ratios in the presence of ADP and $P_i$ but respire only slowly in the absence of ADP. Such "tight coupling" of respiration with mammalian preparations has been observed in many laboratories and has led to the concept that the ADP level is an important determinant of the rate of respiration. On the other hand, insect mitochondria, although capable of yielding high P:O ratios in the presence of ADP, respond somewhat differently to the phosphate acceptor. In early experiments, Sacktor (81) found that the rate of respiration of *Musca* flight muscle mitochondria was independent of the nucleotide concentration. Similar observations were subsequently made with fly mitochondria by Sacktor & Cochran (93) and Chance & Sacktor (17), with locust muscle preparations by Rees (76), and with mitochondria from *Periplaneta* by Cochran & King (26). Dinitrophenol was without effect on the respiration of mitochondria from locusts [Rees (76)] or mature flies [Sacktor & Cochran (89); Slater & Lewis (101)]. These findings are consistent with the absence of ADP stimulation. Remmert & Lehninger (77) have referred to insect mitochondria that respond in this manner as "loosely coupled," contrasting this response with those of other mitochondria described as "tightly coupled" or "uncoupled." These investigators have recently found a protein, called R factor, which is present in liver mitochondria and converts tightly coupled mitochondria to a loosely coupled preparation.

In a few studies, ADP caused relatively small increases in the rate of $\alpha$-glycerophosphate oxidation. For instance, using gently teased flight muscle of *Phormia*, Sacktor (86, 87) found a small, about threefold, stimulation of oxygen uptake. This effect is greater than that observed earlier with ADP and isolated *Musca* flight muscle mitochondria [Sacktor (84)] but is similar in magnitude to that seen previously with dibromophenol [Chance & Sacktor (17)]. Bücher, Klingenberg & Zebe (7) have isolated mitochondria from *Locusta* which show a stimulation of $\alpha$-glycerophosphate oxidase of 2.5 times on addition of ADP; however, in a later publication from their laboratory, Zebe, Delbruck & Bücher (120) state that oxygen intake could not be significantly influenced by the addition of adenine nucleotide. At the same time as this latter paper, another report by Klingenberg & Bücher (52) stated that

"respiratory control" with locust mitochondria oxidizing α-glycerophosphate is seen only when the locust is very young and in a narrow and specific age range. Thereafter, respiratory control with α-glycerophosphate stops, but can still be demonstrated with pyruvate plus malate as the substrate. It should be pointed out that these observed respiratory stimulations by ADP with insect preparations are smaller in magnitude than those observed with mammalian mitochondria and are much too small to account for the physiological control of respiration in the living insect. This aspect of the problem will be discussed later in this review.

Concomitant with measurements of increased respiratory activity of *Musca* mitochondria upon addition of dibromophenol, Chance & Sacktor (17) obtained spectroscopic information which indicated that the uncoupler acts through a "reversal of inhibition mechanism" similar to that described for the mammalian phosphorylation system [Chance & Williams (20)]. Addition of dibromophenol caused oxidation of cytochrome components below *a* and, hence, reversed an inhibition of electron transport through the carriers themselves. This crossover point between cytochromes-*c* and -*a* localizes one site of phosphorylation during the oxidation of α-glycerophosphate. Spectroscopic data using locust mitochondria [Bücher, Klingenberg & Zebe (7); Klingenberg, Slenczka & Ritt (53)] or fly muscle preparations [Sacktor (86, 87)] showed that ADP brings about the same changes in the steady state oxidation-reduction level of the respiratory pigments as seen previously with dibromophenol. This evidence supports the hypothesis that the phosphorylating mechanism of flight muscle mitochondria is dependent upon the action of an electron transport inhibitor such as has been postulated for the mammalian system.

## Cytoplasmic-Mitochondrial Interrelationships

In early experiments, Sacktor (82) outlined the metabolic pathways found in insect flight muscle and showed that these biochemical activities are localized within specific morphological components of the muscle. It was evident from this study, as well as related investigations, that the anaerobic pathway of carbohydrate breakdown, glycolysis, and the aerobic pentose-shunt are in the soluble sarcoplasm, cytoplasm [Chefurka (21, 22); Sacktor (82)], whereas the enzymes participating in the citric acid cycle, α-glycerophosphate oxidase, and the electron transport chain are in the mitochondria [Sacktor (80, 82); Watanabe & Williams (106)].

Sacktor (82) pointed out that the later stages of glycolysis in insect flight muscle were different from the biochemical scheme as found in vertebrate muscle. Of particular importance was the observation that lactic dehydrogenase activity was negligible in *Musca* flight muscle. From this finding, plus other information, such as the imbalance between glycogen breakdown, $CO_2$ evolution, and lactic acid formation in several insect preparations [Barron & Tahmisian (3); Gilmour (37); Humphrey & Siggins (46)], and the absence of

an appreciable oxygen debt in flies even after a prolonged and exhaustive flight [Chadwick & Gilmour (11)], Sacktor (82) postulated an alternative mechanism of glycolysis in flight muscle. Support for this viewpoint came from the finding of an extraordinarily active α-glycerophosphate dehydrogenase in flight muscle [Sacktor & Cochran (91); Zebe & McShan (121)]. This enzyme was found in the cytoplasm of the muscle and was DPN-linked. Comparative studies by Zebe & McShan (121) revealed a negative correlation between the activities of α-glycerophosphate dehydrogenase and lactic dehydrogenase in different kinds of skeletal muscle. Confirming studies by Kubista (54, 55) and Chefurka (23) were important in establishing the generalization that insect flight muscle has insignificant lactic dehydrogenase activity and consequently little lactic acid formation. Instead, there is an extremely active α-glycerophosphate dehydrogenase that catalyzes the oxidation of DPNH while synthesizing α-glycerophosphate. Thus, in flight muscle, carbohydrate is glycolyzed by the pyruvate-α-glycerophosphate dismutation system,

$$\text{Glucose} + \text{P} \rightarrow \text{pyruvate} + \alpha\text{-glycerophosphate.} \qquad 6.$$

When viewed in the light of our early reports that flight muscle mitochondria oxidized α-glycerophosphate at tremendous rates [Chance & Sacktor (17); Chance & Williams (19); Sacktor (84)], the extramitochondrial DPN-linked formation of α-glycerophosphate assumes a new significance. We found that, in the presence of α-glycerophosphate and with the use of the uncoupler dibromophenol to obtain maximum rates, respiratory activity, in terms of $K_{tc}$, reached values as high as 620 and turnover numbers for the cytochromes approached levels calculated for the respiratory pigments during actual flight. In fact, these respiratory rates with isolated fly mitochondria could account for the oxygen uptake of the fly during flight. The two- to fourfold increase in respiration found with dibromophenol [Chance & Sacktor (17)] is also obtainable with the physiological phosphate acceptor ADP [Bücher, Klingenberg & Zebe (7); Sacktor (86, 87)]. Although the exceptionally high respiratory rates with α-glycerophosphate are seen only with optimal conditions of measurement, such as with the rapid oxygen electrode assay, appreciable oxidative rates with this substrate have been found, manometrically, by several investigators, including Watanabe & Williams (106), Zebe (118), Sacktor & Cochran (89, 93), Zebe, Delbruck & Bücher (120), and Cochran & King (26).

Equilibria of the two α-glycerophosphate reactions, as well as an appreciation of the importance of cytological localization and mutual interaction of these enzymes, led to the concept of the α-glycerophosphate cycle [Bücher & Klingenberg (6); Estabrook & Sacktor (29); Sacktor (85); Zebe, Delbruck & Bücher (119)]. This cycle, Figure 2, schematically represents a mechanism whereby hydrogen from the extramitochondrial pool of DPNH is made available to the respiratory chain. DPNH formed by glyceraldehyde phosphate

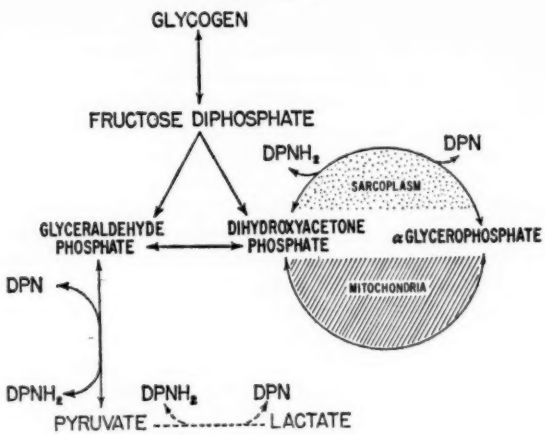


FIG. 2. α-Glycerophosphate cycle.

dehydrogenase during glycolysis is reoxidized by the extramitochondrial α-glycerophosphate oxidation-reduction system:

$$\text{DPNH} + \text{H}^+ + \text{DHAP} \rightleftharpoons \text{DPN} + \alpha\text{-glycerophosphate (cytoplasmic enzyme).} \quad 7.$$

This reaction is linked with the direct mitochondrial oxidation of α-glycerophosphate:

$$\alpha\text{-Glycerophosphate} + \tfrac{1}{2}\text{O}_2 \rightarrow \text{dihydroxyacetone phosphate} + \text{H}_2\text{O (mitochondrial enzyme).} \quad 8.$$

The sum of these reactions represents the oxidation of DPNH by oxygen:

$$\text{DPNH} + \text{H}^+ + \tfrac{1}{2}\text{O}_2 \rightarrow \text{DPN} + \text{H}_2\text{O}. \quad 9.$$

In addition to the α-glycerophosphate cycle, other pathways have been described for the oxidation of extramitochondrial DPNH. The various schemes include: (*a*) the direct mitochondrial oxidation of exogenous DPNH

$$\text{DPNH} + \text{H}^+ + \tfrac{1}{2}\text{O}_2 \rightarrow \text{DPN} + \text{H}_2\text{O};$$

(*b*) the oxidation of DPNH by the cytoplasmic lactic dehydrogenase

$$\text{DPNH} + \text{H}^+ + \text{pyruvate} \rightleftharpoons \text{DPN} + \text{lactate};$$

(*c*) the oxidation of DPNH by the cytoplasmic malic dehydrogenase

$$\text{DPNH} + \text{H}^+ + \text{oxalacetate} \rightleftharpoons \text{DPN} + \text{malate};$$

and (*d*) the oxidation of DPNH by the cytoplasmic α-glycerophosphate dehydrogenase (Reaction 7)

$$\text{DPNH} + \text{H}^+ + \text{dihydroxyacetone phosphate} \rightleftharpoons \text{DPN} + \alpha\text{-glycerophosphate.}$$

Scheme (*a*) and Scheme (*c*), coupled with the mitochondrial oxidation of malate, and Scheme (*d*), coupled with the mitochondrial oxidation of $\alpha$-glycerophosphate (Reaction 8), enable reducing equivalents to pass through the cytoplasmic-mitochondrial barrier; by this barrier we mean the inability of the enzymes of the intact mitochondria to act on specific substrates outside. Scheme (*b*) does not enable hydrogen to pass the barrier; instead, it leads to accumulation of lactic acid.

Studies were initiated in our laboratory and in Bücher's to determine the contributions of each of these routes in hydrogen transport. Sacktor (80) reported the very rapid direct oxidation of exogenous DPNH by *Musca* flight muscle mitochondria washed in physiological saline. Appreciable, although less, DPNH oxidase activity was found when the mitochondria were isolated in isotonic sucrose [Chance & Sacktor (17)]. More recent experiments attempted to reconstruct the processes taking place in the intact muscle [Sacktor (87)]. Flight muscle from *Phormia* was gently and carefully teased, and measurements of DPNH oxidation were started within one minute from the time the muscle was removed from the fly. With this mild treatment, there was no direct oxidation of DPNH in most experiments. In a similar study, Zebe, Delbruck & Bücher (120) reported that homogenate sediments of *Locusta* flight muscle show an active direct oxidation of DPNH and that teased muscle preparations have less activity. However, in contradistinction with the experimental observations with carefully teased blow fly flight muscle, Zebe, Delbruck & Bücher (120) derived a figure of 40 per cent for the share of hydrogen from the extramitochondrial DPNH that is oxidized directly by mitochondria [Scheme (*a*)]. However, these workers admitted that this calculated figure, based in part on an observed direct DPNH oxidation, is probably too high, since the relative impermeability of the cytoplasmic-mitochondrial barrier was not taken into account.

In other experiments with teased *Phormia* flight muscle, the addition of pyruvate had no effect on the oxidation of extramitochondrial DPNH [Sacktor (87)]. This confirms the insignificance of lactic dehydrogenase in fly flight muscle [Sacktor (82); Zebe & McShan (121)].

In sharp contrast to this veritable absence of Schemes (*a*) and (*b*), Sacktor (87) reported that the addition of dihydroxyacetone phosphate to reaction mixtures containing as little as 100 $\mu$g. muscle/ml. caused an oxidation of all the DPNH in less than one minute. In further studies, Sacktor & Dick (94) have found that in the presence of antimycin-A, added to prevent the mitochondrial oxidation of $\alpha$-glycerophosphate, dihydroxyacetone phosphate brought about a stoichiometric oxidation of exogenous DPNH. When catalytic amounts of dihydroxyacetone phosphate were added in the absence of antimycin-A, thus allowing the mitochondrial oxidation of $\alpha$-glycerophosphate and thereby making more dihydroxyacetone phosphate available for the extramitochondrial DPNH [Sacktor & Cochran (92)], all the excess exog-

enous DPNH was oxidized. These data provide an *in vitro* demonstration of the α-glycerophosphate cycle. In addition, the data suggest that, in flight muscle of flies, oxidation of the extramitochondrial pool of DPNH is mediated for the most part by the α-glycerophosphate dehydrogenase [Reaction 7, Scheme (d)] and that this enzyme in combination with the mitochondrial oxidation of α-glycerophosphate (Reaction 8) provides the major vehicle for passing the hydrogen of DPNH through the cytoplasmic-mitochondrial barrier. Zebe, Delbruck & Bücher (120) calculated a value of 60 per cent for this pathway, but, in this figure, as in the direct oxidative pathway [Scheme (*a*)], permeability considerations were not taken into account.

The malate-oxalacetate system [Scheme (*c*)] may function in a manner similar to the α-glycerophosphate-dihydroxyacetone phosphate system for oxidizing exogenous DPNH. Sacktor (80) showed that *Musca* flight muscle contained both a mitochondrial and an active cytoplasmic malic dehydrogenase. Recently, Delbruck, Zebe & Bücher (27) reported that the two malic dehydrogenases in *Locusta* are distinct enzymes. They postulated that the malate-oxalacetate redox reactions have an equalizing function rather than functioning as a constant hydrogen transport in one direction as is the case in the α-glycerophosphate-dihydroxyacetone phosphate system. In teased flight muscle of *Phormia*, Sacktor & Dick (94) found that, in some experiments, oxalacetate addition caused an oxidation of extramitochondrial DPNH at a rate about 25 per cent that obtained with dihydroxyacetone phosphate. However, it was noted that the rate of DPNH oxidation by malic dehydrogenase was markedly influenced by the concentration of coenzyme and substrate and that any comparison of the two enzymic activities must take these variables into consideration. It should be pointed out that, as shown in Table IV, the rates of oxidation of α-glycerophosphate and malate by fly flight muscle mitochondria are in different orders of magnitude. This would support the viewpoint of Delbruck, Zebe & Bücher (27) on the functional difference between the two redox systems.

## Control of the Respiratory Activity

In the intact insect, the increase of respiratory rate upon initiation of flight is often about 50:1 and can be as high as 100:1. Thus, the absence of any, or occasionally the slight, stimulatory effects of ADP or other nucleotides upon the respiratory rate of flight muscle mitochondrial suspensions raised some questions about the physiological control mechanisms involved [Chance & Sacktor (17); Estabrook & Sacktor (29); Sacktor (85); Sacktor & Cochran (93); Zebe, Delbruck & Bücher (120)]. The only evidence for respiratory control in insect mitochondria, oxidizing α-glycerophosphate or citric acid cycle intermediates, is the two- to fourfold increase in rate of oxygen uptake obtainable with the uncoupling agent, dibromophenol, or, in some instances, with ADP [Bücher, Klingenberg & Zebe (7); Chance & Sacktor (17); Sacktor (86)]. Such increases due to ADP addition, however,

in contrast to those found in frog muscle [Chance & Connelly (16)], are not of sufficient magnitude to satisfy the requirements for a physiological control mechanism in an insect just beginning flight.

Although it is possible that this ADP effect represents a remnant of the physiological control mechanism damaged by our preparative methods, microscopic examinations of our teased preparations, which have a response to ADP similar to that of isolated mitochondria [Sacktor (86, 87)], showed that many of the mitochondria were still in a position adjacent to the myofibrils. The observation that exogenous DPNH was not oxidized directly [Sacktor (87)] indicated that the mitochondria in the teased muscle preparations retained a critical selective permeability, which often characterizes undamaged mitochondria. Thus, it appeared to us unlikely that respiratory control would have been lost with such mild treatment. We proposed the theory that respiratory control in flight muscle of flies may not only be caused by control of the phosphate acceptor or $P_i$ concentration but also by regulation of substrate concentration or $\alpha$-glycerophosphate oxidase activity [Chance & Sacktor (17); Estabrook & Sacktor (29); Sacktor (85, 87)].

Other evidence pertinent to this concept is now available. Chance & Sacktor (17) showed that $\alpha$-glycerophosphate is the only known substrate to which the mitochondria exhibit sufficient activity to sustain the respiratory activity in flight. The lesser activities with other substrates, for example the Krebs cycle acids, are noteworthy. At most, the rates of oxidation with these intermediates account for the respiration of the fly at rest. The fact that the rate of $\alpha$-glycerophosphate oxidation exceeded that obtained with succinate by a factor of 10 to 20 and with other intermediates by factors as large as 50 to 100 suggests that, on a kinetic basis, the Krebs cycle does not mediate a significant portion of the flight muscle metabolism. Obviously, one can not conclude that this hypothesis requires that the Krebs cycle functions only when the fly is at rest and that it ceases to function when the fly initiates flight.

The high rate of oxidation of $\alpha$-glycerophosphate by fly flight muscle mitochondria relative to that of other substrates supports the idea first suggested by Chance & Sacktor (17) that $\alpha$-glycerophosphate is the physiological substrate for the activated mitochondria. A very different explanation for the high activity of $\alpha$-glycerophosphate oxidase has been given by Bücher, Klingenberg & Zebe (7). In contrast to the findings of Chance & Sacktor (17) that in fly mitochondria the turnover number of the cytochromes during the oxidation of $\alpha$-glycerophosphate reached the turnover number of the respiratory enzymes during flight, Bücher, Klingenberg & Zebe (7) reported that in locust mitochondria the activity of $\alpha$-glycerophosphate oxidase is only 20 per cent that of the turnover number for cytochrone-*c* during flight of the locust. A combination of Krebs cycle intermediates, such as pyruvate plus malate, yields a turnover number of only 10 per cent the flight rate. Yet, these investigators suggest that the high relative rate of $\alpha$-glycerophosphate oxidation is attributable to the loss of activity with

Krebs cycle substrates without the impairment of the rate of α-glycerophosphate respiration. They argue that loss of activity with Krebs cycle intermediates is the result of the loss of mitochondrial DPN that occurs when mitochondria are damaged and aged excessively. They further suggest that the low rates of Krebs cycle oxidation by fly mitochondria found by Chance & Sacktor (17) was the result of damaged preparations. (It must be assumed that this applies to their preparations of locust mitochondria as well, although not to the same extent.) This viewpoint does not take into account the following facts. First, even if the oxidation of Krebs cycle intermediates by fly mitochondria were impaired, this would not increase the activity of α-glycerophosphate oxidase, which Chance & Sacktor (17) showed was great enough to approach the rate of respiration during flight. Second, while the loss of mitochondrial DPN undoubtedly can cause a decrease in the rates of oxygen uptake of some citric acid cycle intermediates, this loss should not affect the oxidation of succinate, which is an important member of the Krebs cycle but, like α-glycerophosphate, does not include pyridine nucleotide in its direct respiratory chain (see Figure 1). However, the oxidation of α-glycerophosphate by fly mitochondria was 10 to 20 times that of succinate. On the other hand, succinate oxidation was equal to that of α-ketoglutarate, a Krebs cycle intermediate requiring DPN, but decidedly better than other DPN-requiring substrates [Chance & Sacktor (17)]. Thirdly, recent studies by Sacktor (88) with teased flight muscle of *Phormia*, which exhibit as great an ADP effect as locust preparations, showed that the rate of oxidation of α-glycerophosphate is in the same relation to the rates of oxidation of citric acid cycle members and endogenous substrates as found with isolated fly mitochondria. As pointed out before, in these teased preparations a large number of the mitochondria remain attached to the myofibrils, and respiratory measurements are completed within, at most, five minutes of their removal from the fly. The nature of any damage is obscure and to me its existence seems quite unreasonable.

Strong support for the importance of α-glycerophosphate in flight metabolism also comes from reports of very high concentrations of this substrate in insect tissues. For instance, over 60 per cent of the total acid soluble phosphate in blood of *Cecropia* was identified as α-glycerophosphate [Wyatt (116)]. Winteringham (110, 111) has compared the distribution of total soluble P in flight muscle of house flies at rest and in full flight. He found that within a few seconds of the initiation of full flight the concentration of α-glycerophosphate fell drastically from a high level of 23 per cent of the total soluble P to half this value. A similar decrease in α-glycerophosphate occurred on initiation of flight in *Locusta* [Bücher & Klingenberg (6)]. These results demonstrate that, in the living insect, α-glycerophosphate is available for utilization and is rapidly oxidized to meet the immediate demands of a sudden flight.

Our previous study suggested that control of α-glycerophosphate oxidase activity can provide a mechanism for respiratory control [Estabrook &

Sacktor (29)]. It was found that the chelator, ethylenediamine tetraacetate (Versene®), blocked α-glycerophosphate oxidation and that this inhibition was reversed by divalent cation or additional substrate. If one presumes that flight muscle contains an endogenous chelator capable of functioning in a manner analogous to ethylenediamine tetraacetate, then still another system for control can be conjectured. In this system during rest, α-glycerophosphate oxidase is in an inhibited state, and maintenance activity and the low level of respiration observed at rest is achieved by the Krebs cycle. In flight, this inhibition is reversed, either by an accumulation of substrate or by divalent cations, such as magnesium, released during nervous stimulation of the muscle. This reversal results in the high respiratory rate characteristic of α-glycerophosphate oxidation. This scheme is compatible with the findings that α-glycerophosphate levels were high during rest and that they declined rapidly when flight was initiated.

As suggested by Chance & Sacktor (17) earlier, spectroscopic studies of the intact thorax during flight could resolve the question of whether or not respiratory control in insect mitochondria is exerted at the substrate level. If, on initiation of flight, cytochromes-*b* and -*c* of the aerobic muscle become more oxidized, control is exerted through the phosphate acceptor. If, however, cytochromes-*b* and -*c* become more reduced, then the control is probably exerted through the substrate. Keilin's (49) early experiments on the wax moth suggest that the latter hypothesis is correct, although direct measurements of these events in other insects are obviously required.

If control of flight respiration in flies is largely caused by regulation at the substrate level, a logical site for control would be the formation or oxidation of α-glycerophosphate. Nevertheless, alternative loci must be considered. Although the catabolism of hexoses is fairly well defined, we know relatively little about the factors regulating the breakdown of the carbohydrates, glycogen and trehalose, to hexoses in insect tissues or about their reactions. α-Glucosidases, which hydrolyze α-glucosides to utilizable hexoses, have been reported from *Musca* flight muscle [Sacktor (82)]. The enzyme trehalase has recently been found; it specifically catalyzes the hydrolytic cleavage of trehalose into two glucose moieties [Zebe & McShan (122)]. In investigations still in progress, Friedman (34) observed that *Phormia* possess an endogenous inhibitor of trehalase activity. While we await further characterization of this important inhibitor from his laboratory, one may speculate on a role for this substance in regulating the concentration of substrate available to the respiratory enzymes. In fact, one may even visualize a multifaceted respiratory control system in which the concentration and metabolism of crucial substrates and their precursors are regulated at several critical sites, including α-glycerophosphate, trehalose, $P_i$, to a limited extent ADP, and others yet to be described.

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# DIURNAL RHYTHMS[1]

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## Introduction

The occurrence of diurnal rhythms throughout the animal and plant kingdoms is now a well-known and well-described phenomenon. Until fairly recently these rhythms were known only as the result of field observations on the timing of various activities. The discovery that such rhythms may continue when an animal or plant is placed in constant environmental conditions initiated a new stage in the study of the problem and has given rise to a new terminology. The term "diurnal rhythm" is still in general use, but "twenty-four hour rhythm" is common, although open to objection on the grounds that the period of the rhythm is rarely exactly 24 hours. As an answer to the latter objection, a new term, "circadian rhythm," is gradually being introduced.

Whichever term is used, it generally implies that the rhythm is one that will persist for at least two or three days in a constant environment, that it is little affected by temperature within normal biological limits, and that the timing of the peaks or phases of the rhythm in relation to solar time is originally determined by an environmental change, such as a variation in light intensity or temperature.

A number of reviews covering all or parts of the animal kingdom have appeared recently [Bruce & Pittendrigh (10); Bunning (11); Harker (43); Webb & Brown (91)]; therefore only recent work is reviewed here, except where it is felt that attention need be drawn to the older literature or where reference to it is necessary in relation to new work.

## Persistence of Rhythms

Basic to the modern concept of diurnal rhythms is the continuance of the rhythm for at least a few days in constant environmental conditions. The length of time a rhythm persists varies from several days, for the majority of animals, to three months or longer in a few cases, for example, in mayfly nymphs [Harker (39)] and at least one beetle, *Bolitotherus cornutus* (Panzer) [Park & Keller (73)]. The rhythm may even persist through the major physiological and anatomical changes of metamorphosis (24), as has been recorded in *Bolitotherus* and in the Queensland fruit fly *Dacus tryoni* (Froggatt) [Bateman (2)]; indeed, in the latter the rhythm may even be maintained from one generation to the next. It is possible that some of the rhythms recorded as being persistent over very long periods may have inadvertently been reinitiated, for work discussed later in this review shows that extremely short flashes of light will initiate a rhythm in *Drosophila*.

[1] The survey of literature pertaining to this review was concluded in March, 1960.

When an animal is kept in constant light or darkness, the period of the persisting rhythm is rarely, if ever, exactly 24 hr., and it is this characteristic that is most frequently cited as evidence for the presence of an internal control or "clock." The length of the period in continuous darkness has been called the natural period, or free-running period, of the particular animal [Pittendrigh & Bruce (77)]. As it seems that the environmental conditions determine the period length, within the limits offered by the genetic constitution of the individual [Harker (43)], it is perhaps misleading to designate any exact time as the natural period.

## Phase Setting

The phases of any diurnal rhythm may be set in relation to solar time by a variety of environmental factors. A change in light intensity is the most powerful phase setter in practically every case. For example, the active phase of the cockroach *Periplaneta americana* (Linnaeus) occurs during the night, even in widely fluctuating temperature conditions, but the active phase is moved to the daytime if the light period is given during the night and darkness during the day [Harker (42)]. The phase shift is maintained for some time in constant conditions. A distinction should be drawn between the effect of a change of light intensity on an established rhythm and the effect on an animal that is showing no measurable rhythm.

*Initiation.*—A rhythm may be initiated in an arrhythmic animal by a short, unrepeated light period. For the cockroach, the length of the light period necessary to establish an activity rhythm is about 2 hr. [Harker (42)], but in *Drosophila* an eclosion rhythm can be initiated by a flash of light of only 1/2000 sec. [Pittendrigh & Bruce (78)]. A period of darkness after continuous light is as an effective an initiator as the reverse condition, and a period at a higher or lower light intensity, provided the intensity change exceeds a threshold value, will also initiate a rhythm. The form of the rhythm may vary with the type of environmental change. This feature has attracted little attention and is one that might provide useful information about the nature of the internal clock. When *Periplaneta* is given one dark period after continuous light, the peak of activity occurs toward the beginning of the dark period; but, when a light period is given after continuous darkness, two peaks of activity appear, one at the beginning of the light period and one at the beginning of the new dark period. In the latter case, the two peaks do not persist for similar periods; the peak occurring in the light phase fades some days before the other [Harker (43)]. When *Drosophila* cultures are transferred from continuous light to darkness, the new peak of eclosion occurs 36 hr. after the transfer; the insects react as though the change from light to darkness were the time of "sunset," and the time allowed for a dark period before the beginning of eclosion is related to the expected time of "sunrise" [Pittendrigh & Bruce (78)].

A rhythm can be initiated by temperature fluctuations, the relationship

between the phases of the rhythm and the time of the temperature fluctuations varying with the species. Temperature fluctuations do not appear to act as strongly or as generally as do light fluctuations, but temperature-dependent rhythms have been shown to exist in *Periplaneta* [Cloudsley-Thompson (18)], *Ptinus* [Bentley, Gunn & Ewer (4)], *Ephestia* [Scott (86); Moriarty (65)], and *Drosophila* [Pittendrigh & Bruce (77)].

*Perturbation of an established rhythm.*—When a diurnal rhythm is already clearly established after an insect has been kept in alternating environmental conditions, a change in the timing of the environmental fluctuations might be expected to affect the phases of the diurnal rhythm, but probably not to reset them completely.

A number of studies dealing with the effect of a perturbation in the environmental cycle on the eclosion rhythm of *Drosophila* have been carried out by Pittendrigh and his associates [Pittendrigh (76); Pittendrigh & Bruce (77, 78); Pittendrigh, Bruce & Kaus (79)]. Light periods of 12 hr. were given to each of 23 cultures of *Drosophila*, the light period for each starting at a different time of day. The intervals between the peaks subsequent to the light period were either longer or shorter, depending upon the solar time when the light period occurred. When the new light period fell within 15 hr. of the previous eclosion peak, successive peaks were more than 24 hr. apart; but, when the light period occurred more than 15 hr. after the previous emergence peak, the subsequent peaks were less than 24 hr. apart. Seventy-two hours after the beginning of the experiment, the phases of all the cultures had shifted to lie in phase with their light signal. These results are of considerable interest, for they show that the attainment of the new phase is not instantaneous, but that the endogenous controlling system goes through several transient cycles with periods differing from those of the normal steady state.

The experiments were repeated with a 4 hr. light period, and, although a resetting of phase finally occurred, the transient cycles followed a different course from those in the previous experiment. When the 4 hr. light period came close to the time of the previous peak, there was little immediate effect on the rhythm, whereas, when the light period came about 15 hr. after the previous peak, the next peak was delayed considerably. If the light period occurred still later, successive peaks followed rapidly, two or three occurring in 24 hr. The length of the photoperiod obviously affects whatever mechanism is concerned with phase shift. In this connection, it is of interest to consider another observation of Pittendrigh & Bruce (78); they found that, when a 4 hr. light to 20 hr. darkness cycle was continued, the peak of eclosion occurred at the end of the dark period, not at the beginning of the light period as is the case with other photoperiods. The intensity of the light also affects the final setting of the phase.

Two features of the above experiments need to be emphasized. Firstly, it must be remembered that, although the transient cycles represent the

reaction of the culture as a whole, each animal is featuring only once; the peak of emergence occurring 48 hr. after the light period was produced by animals which were, when the signals occurred, at a different stage of development from that of those animals which produced the peak at 72 hr. Secondly, there is no reason to suppose that only the change from darkness to light is important in phase setting; the different results obtained after each of the two lengths of photoperiod may be as much related to the time of the return to darkness as to the actual length of the light period.

The effect of an alteration in the environmental light cycle on the activity rhythm of *Periplaneta* has been observed [Harker (45)], and the time of the subsequent activity peaks has been related to the effect of a change from light to darkness on the secretory cycle of those suboesophageal ganglion cells on which the activity rhythm is dependent (see p. 138). There appears to be a stage in the neurosecretory cycle when secretion will take place whatever the light condition, a stage when secretion can take place if there is a change from light to darkness, and a stage when no secretion will take place whatever the light conditions. Immediate resetting of the phases of the neurosecretory cycle occurs only when a change from light to darkness coincides with the "possible secretion" stage. When the light is turned off during the "impossible secretion" stage, there is no immediate effect on the cycle, but the next episode of neurosecretion is brought forward by 4 to 6 hr. Because of the different effects of a change from light to darkness on the different stages of the neurosecretory cycle, the activity peaks appearing after such a change may be more, or less, than 24 hr. apart, so that the transient cycles observed are similar to those appearing in the *Drosophila* eclosion rhythm. As opposed to the above results, the active phases of *Gryllus domesticus* Linnaeus return to their previous positions if the insect is kept under new environmental conditions for only a short time [Lutz (57)].

Temperature fluctuations will affect an established eclosion rhythm in *Drosophila*. Transient peaks occur, but the final setting of the phases shows relatively little change from their previous times [Pittendrigh & Bruce (77)]. The eclosion rhythm of *Ephestia* appears to be largely controlled by temperature, and, given a constant temperature, Scott (86) was unable to reverse the phases of the rhythm by reversing the light-to-darkness phases.

A change, during the life cycle, of sensitivity to one type of environmental factor from another has been recorded in the fruit fly *D. tryoni* [Bateman (2)]. These flies show a diurnal rhythm of eclosion that is persistent in constant conditions from one generation to the next. The phases of the rhythm can be reset in the larval stage by new light:darkness conditions, but the pupae are insensitive to such changes and react only to temperature fluctuations.

*Oscillator model.*—Pittendrigh & Bruce (77) have postulated a model of the internal controlling system which would consist of one or more endogenous self-sustaining oscillators that have a natural period close to 24 hr.,

are temperature-independent, and in which both phase and period are susceptible to entrainment by one another or by other systems (e.g., light cycle). When the organism is in constant light and temperature, such an oscillator is free-running and reveals its natural period, and its phases can be shifted by single non-periodic signals. The authors describe the transient effects that follow the new timing of a light period as the transient motion of perturbed oscillations before a new steady state is achieved.

*Timing of phase in relation to environmental signals.*—Just as the period of a diurnal rhythm appears to lie within a given range for a species, with the individuals varying within that range, so the times at which the phases of a rhythm occur in relation to the entraining environmental cycle vary specifically and individually. *Drosophila* eclosion peak, for example, occurs at, or soon after, a change from darkness to light, whereas the peak of activity in *Periplaneta* comes soon after the change from light to darkness. Variation in phase position may appear in some environments, however, as has been observed in *Drosophila*, which shows its main emergence peak before dawn if the light period is only 4 hr.

In a few cases, variation within a species has been recorded as being genetically produced. In the butterfly *Colias*, females differing by one sex-linked gene are reported to fly at different times [Hovanitz, quoted in Kalmus (53)]. Various mutants of several *Drosophila* species emerge at characteristically different times [Kalmus (53)].

The males and females of many species consistently show a time difference in emergence peaks or peaks of attraction to light (p. 142); this would seem to have a genetical basis. An interesting observation has been made on the activity times of groups of *Drosophila:* when groups are monosexual two peaks of diurnal activity occur, whereas bisexual groups show only one activity peak and that at night [Ohsawa *et al.* (68)]. Lengthening of the period between the environmental "signal" and the peak of activity seems to occur with age in many insects. Further studies on these phenomena are badly needed.

The number of animals in a population may affect the phase position of rhythms. The time of emergence of *Drosophila* becomes later each day as the population increases [Mori (64)], and Park (71) found that the time of activity of the beetle *Megalodacne* varies with the number of animals in the group. Williams (94) observed that the beetle *Feronia madida* (Fabricius) showed a 12 hr. shift in phase in different habitats; it was active only during the day in open ground and only at night in woodland.

The interaction of two environmental cycles may alter the time of the peak of activity, as has been shown in the ant *Messor semirufus* André [Bodenheimer & Klein (7)]. In normal alternating light and darkness and low temperatures this ant is most active at noon; at slightly higher temperatures it is active during the evening; and at still higher temperatures it is active at night.

### Environmental Cycles Other than Twenty-Four Hour Cycles

The effect of keeping animals in environmental conditions that fluctuate rhythmically, but have a period greater or less than 24 hr., has been studied by a number of workers. In the main, these studies have been designed to test the permanence of the 24 hr. rhythm and to see whether rhythms of other periods could be imposed upon and retained by the animal.

As the effect of one light period at an unusual time is to produce transient peaks (p. 133), it is not unexpected that environmental cycles differing from 24 hr. have a complex effect on insects.

The beetle *Anthea venator* Fabricius kept in a 9 hr. light to 9 hr. darkness cycle takes up an activity rhythm with an 18 hr. period, but this does not persist in continuous darkness, the insect reverting to a 24 hr. rhythm [Cloudsley-Thompson (19)]. Neither *Blaps requieni* Solier nor *Periplaneta* will take up an 18 hr. rhythm [Cloudsley-Thompson (18, 19)].

When *Periplaneta* is kept in a 2 hr. light to 6 hr. darkness cycle, its activity occurs, for a few days, during the light period, and then it becomes completely arrhythmic. There is no reversion to a 24 hr. period when it is placed in continuous darkness, and, if it has experienced the 8 hr. cycle for about ten days, it will no longer take up a 24 hr. rhythm even in a 12 hr. light to 12 hr. darkness situation. Major pathological changes occur in the gut after this treatment, even if food is only given at 24 hr. intervals during the 8 hr. environmental cycle (Harker, unpublished). When the effect of one light period on the various stages of the neurosecretory cycle are considered (p. 134), it can be seen that in a cycle of less than 8 hr., the light period is constantly resetting the neurosecretory cycle. It is possible that the secretion may be finally exhausted in these conditions. The pathological effects may perhaps be related to those occurring when suboesophageal ganglia secreting 12 hr. out of phase with the animal's own ganglion are implanted into cockroaches. After such treatment animals develop malignant tumours [Harker (44)].

The rhythm of *Drosophila* eclosion conforms to a 12 hr. environmental cycle when the insects are kept in a 6 hr. light to 6 hr. darkness ratio, but the rhythm returns to a 24 hr. periodicity when the animals are placed in continuous darkness; the phase setting is then determined by the time of the last light period [Brett (8)].

Remmert (83) kept the chironomid *Pseudomittia arenaria* Strenzke, which has a diurnal rhythm of eclosion, in light cycles of 6, 12, 18, 24, 28, 36, 42, 54, and 72 hr. The insects conform to the 18, 24, and 28 hr. cycles. Two peaks occur in the 42 and 48 hr. cycles. In cycles longer than 48 hr. or shorter than 12 hr. emergence becomes random. In a 12 hr. cycle, the 24 hr. rhythm is maintained, but the insects from some cultures emerge at one of the changes from light to darkness, and the insects from other cultures emerge at the other change. Two peaks of emergence in 24 hr. are obtained only when 6 hr. of

bright light are alternated with 6 hr. of dim light. Bruce & Pittendrigh (10) consider the 12 hr. rhythm a consequence of the fact that the period of the entraining environmental cycle is a submultiple of the natural period of the rhythm, a feature of rhythms which they term "frequency demultiplication." But, as Webb & Brown (91) point out, it seems more likely to be the result of a mixed population with one group 12 hr. out of phase with the other. Such individual differences in phase positions have been found in the rhythms of both activity and oxygen consumption in the grasshopper *Romalea microptera* (Serville) [Fingerman, Lago & Lowe (30)]. A natural population of these insects included individuals showing the peak of activity in the afternoon and others showing the peak in the forenoon.

The eclosion rhythm of *Ephestia* will follow a 16 hr. temperature cycle [Scott (86)] but reverts to a 24 hr. cycle in constant temperature, the phase of the rhythm being determined by the time of the last temperature decrease [Moriarty (65)].

### Continuously Consulted Clocks

The diurnal rhythms so far discussed have all been in the nature of the repetition of an event at approximately 24 hr. intervals. The discovery that bees will return at a precise time to a feeding station discovered the previous day and will do so at any time of day and several times a day [Beling (3); Wahl (90)] suggests the presence of an internal clock, which can be used as a chronometer and not just as an interval timer. The time-sense is not affected by temperature within the range 18°–32°C. [Wahl (90)], nor can it be affected by feeding the bees thyroxin to raise the metabolic rate or quinine to lower it [Renner (84)]. Renner (85) investigated the relationship between the environment and the time-sense by training bees to collect at fixed times and then flying them overnight through 48° longitude, so that the local time difference between the old and new locations was 3 hr. 15 min. In the new locality, the bees showed two searching periods on the first day, the first being related to the original training time, and the second appearing 1½ hr. later. On the second day, the two searching periods were 3 hr. apart; by the third day the two were 3½ hr. apart, which corresponded roughly to the difference between the local time and the old time. The second searching period seems to have been affected by exogenous factors, while the first may have been controlled by the "internal" clock.

Kalmus (52) has shown that the time sense of bees, like the diurnal rhythms of other animals, is delayed when the insects are chilled below 7°C.

*Orientation.*—Animals that orientate by celestial navigation must be able to gain information about time; in this regard, it seems necessary to postulate the presence of a continuously consulted clock similar to that discussed in the previous section.

*Velia*, the pond skater, changes its angle of orientation to an artificial

sun, according to the time of day; that is, it shows a diurnal rhythm of orientation [Birukow (5, 6)]. When kept in constant light or darkness, it loses the orientation rhythm after a time. *Velia* also shows a diurnal rhythm of geotaxis orientation, and the phases of the rhythm can be set by alternating light and darkness. Emeis (27) found the rhythm to be dissimilar to most others in that anaesthesia does not appear to stop the internal clock; insects anaesthetized in the morning act as though kept in constant darkness, and those anaesthetized during the night retained the normal phase positions the next day. Lowering the temperature to 1°–2°C. for 4 hr. also fails to alter the phase positions. An internal time-compensating mechanism has also been found in the ant *Formica rufa* Linnaeus [Jander (50)].

## Physiology

The physiological mechanism of the "internal clock" is as obscure in insects as it is in other animals. Recent writers have tended toward the theory that there are many internal clocks in the more complex animals and that all or any cell may possess the property of acting as a clock [Harker (43); Pittendrigh (76); Pittendrigh & Bruce (77)]. Brown and his associates maintain that the over-all control of diurnal rhythms is dependent on external environmental factors [Webb & Brown (91)] but that an internal clock of some sort is also present.

It is possible that, in insects and other specialized animals, certain functions may be controlled by specific groups of cells forming clocks that are relatively independent of each other (48, 49). One such clock has been found in *Periplaneta*. Neurosecretory cells in the suboesophageal ganglion have been shown to secrete at approximately 24 hr. intervals, and these cells maintain their secretory rhythm for several days when a ganglion is implanted into another animal. A rhythmically secreting ganglion implanted into a headless cockroach induces a rhythm of locomotor activity [Harker (40, 41, 42)]. The timing of the phases of the rhythm appears to be established, through the medium of the ocelli, by a change from light to darkness. Resetting of the phases is not possible at all times of day (see p. 134). There is a second 24 hr. clock in *Periplaneta* which can act on the suboesophageal ganglion neurosecretory cycle and reset the phases of the latter. The neurosecretory cells can be chilled *in situ* and the secretory cycle stopped [Brown & Harker (9)]. The secretory cycle restarts when the animal is warmed, but the phases are delayed by a period equivalent to the period of chilling. While the suboesophageal ganglion is chilled, the phases of the second clock can be reset by a change from light to darkness, and, when the neurosecretory cycle restarts, its phases may, in turn, be reset by the second clock [Harker (46)]. The phases of the second clock may be reset at any time by even a small change in light intensity, but, in the normal animal, the suboesophageal ganglion cycle cannot be reset to times far removed from its previous setting, except by repeated environmental signals. The interaction of the two cycles prob-

ably allows for resetting of the activity rhythm by changes in day-length but prevents resetting by abnormal changes in light intensity during the night, such as might occur in bright moonlight.

Klug (54) found that changes in the nuclear volume of the corpora allata cells in *Carabus nemoralis* Mueller are correlated with the time of locomotor activity, and he suggested that the secretory activity of these cells is related to the activity of the beetle. This still remains to be proven, however, for cellular changes following a 24 hr. cycle have been recorded in other animals [Harker (43)], in plants [Bunning & Schöne-Schneiderhöhn (15)], and in tissue culture [Enderle (28)], and there is no evidence that the corpora allata determines the locomotor activity rhythm.

The 24 hr. activity rhythm of the grasshopper *Romalea* persists after allatectomy [Fingerman, Lago & Lowe (30)], but the length of persistence was not recorded. The rhythm also persists in the absence of the brain, although the level of activity is lower and the peak of activity occurs earlier in the day. In the absence of the suboesophageal ganglion, the rhythm disappears, but, unlike *Periplaneta*, implantation of another ganglion does not evoke a rhythm. This may be attributable to the fact that the brain, corpora allata and corpora cardiaca were still present in the *Romalea* experiments, for recent work [Milburn, Weiant & Roeder (62)] has shown that the corpora cardiaca may affect the nervous activity of the thoracic ganglia. In the experiments on *Periplaneta*, in which a rhythm was induced by an implanted suboesophageal ganglion, either no head was present, or the rhythm had completely disappeared before implantation took place.

Eidmann (26) found that after extirpation of the suboesophageal ganglion, or brain, *Carausius morosus* Brauer became arrhythmic (immobile?) and that reimplantation of the brain did not produce a diurnal rhythm.

*Temperature effects.*—The mixture of temperature-dependence and -independence of diurnal rhythms is one of their most interesting features. The timing of the phases of various rhythms may be temperature-dependent, and yet the period of the rhythm is remarkably temperature-independent. Bunning (12, 14), for instance, has shown that the period of the activity rhythm of *Periplaneta* is temperature-independent between 19° and 29°C.; yet the phases of the activity rhythm can be reset by temperature fluctuations, provided the light is held constant [Cloudsley-Thompson (19)]; on the other hand, if the insects are in alternating light and darkness, even a temperature increase of 10°C. for 4 hr. every 24 hr. will not affect the phase setting [Bunning (14)].

Reducing the temperature to near 0°C. stops the functioning of the clock completely, as has been shown in many animals, including bees [Kalmus (52); Renner (84)] and *Periplaneta* [Harker (45)]. The phases of the rhythm, when the animal's temperature is raised, are delayed by a period equivalent to the length of the chilling.

Pittendrigh & Bruce (78) interpret these temperature characteristics in

terms of their oscillator model and suggest that there are two oscillators, one temperature-independent, which drives a second, which is temperature-dependent. They suggest that the sharp transients produced in *Drosophila* rhythms by temperature decrease reflect the lengthening of the period of the temperature-dependent oscillator and that this oscillator is rapidly re-entrained by the temperature-independent one. Since the latter can only be entrained by light changes, if insects are kept in constant light, the temperature-dependent oscillator can respond to temperature fluctuations and, through a feedback system, reset the other one.

Bunning (14) also subscribes to an oscillator theory and suggests that the cycles of endogenous rhythms are the periods of a relaxation oscillator. He shows that, after *Periplaneta* has been chilled for a given period, the same period at a normal temperature must elapse before a new maximum in the cycle occurs. This implies that the oscillator is not fixed in the prevailing phase by a low temperature but relaxes to its zero position [Bunning (11, 13)]. As there seems to be some disagreement about the effect of chilling on the subsequent phase setting, more evidence is needed on this point.

*Anaesthetics.*—Modification of the activity rhythm of *Periplaneta* has been induced by treatment with carbon dioxide and nitrogen [Ralph (80)]. Two half-hour doses of carbon dioxide are said to reduce the rhythm or to derange it permanently. The effect of nitrogen on the rhythm depends on the stage in the rhythm at which it is given. If the animal is exposed to nitrogen 2 to 4 hr. before the activity peak, there is no effect on the phases of the rhythm, but, if it is given 10 or 22 hr. before the activity peak, the subsequent peak is delayed; the shorter the period between the nitrogen treatment and the next expected peak, the less the delay.

Recent work on *Periplaneta* indicates an interaction between several endocrine systems and the suboesophageal ganglion neurosecretory cycle. These systems are active just after the main peak of locomotor activity (Harker, unpublished) when the animal is sensitive to nitrogen. This suggests that the effect of nitrogen on the activity rhythm is indirect, acting through these subsidiary cycles.

## Field Work

The greatest volume of evidence for the existence of diurnal rhythms under natural conditions comes from observations of flight times, biting cycles, and oviposition cycles. Criticism can be made of any of the methods by which these rhythms are measured on the grounds that any type of trapping or counting may reveal only one particular phase of the general activity pattern. This criticism is mainly relevant, however, when the interpretation of one aspect of behaviour is important, as, for instance, in studies of biting times of mosquitoes in relation to disease control. On the other hand, in general studies of diurnal rhythms, misinterpretation of data may cause the investigator to overlook the interdependence of two types of activity, each

of which is dependent on different environmental conditions. This is an aspect that has been badly neglected and might be a fruitful field for further work. In general, however, whatever the interrelations of the various events of the general activity pattern, there is obvious advantage in selecting any phase that can be measured quantitatively.

Most of the early references to flight activity have been listed by Rau & Rau (81, 82). By far the most comprehensive survey of flight times has been made by Williams (92, 93), who trapped insects at a light over a four year period and found that, although there is a species variation in flight times, most of the species of any one order flew within a given range of times.

The main interest of all the field work on activity rhythms lies in the analysis of the factors that serve as signals in determining the setting of the rhythm phases within the 24 hr. period. Change in light intensity is the main factor affecting phase position in both nocturnal and diurnal animals, as might be expected from the experimental results described previously; as might also be expected from these results, it is rare for activity to occur as an immediate reaction to change in light intensity.

*Anopheles*, in general, has only one peak of activity, which occurs during the dark period [Muirhead-Thompson (66)], and this is related not only to the beginning of the dark period but also to the time of moonlight. The biting activity may be greatest an hour or two later than the maximum flight activity [Halcrow (38)] and may be concentrated into less than an hour [Haddow (34); Haddow, Gillett & Highton (37); Lumsden (56); Mattingly (59)]. An interesting feature, which has received little comment, is the change with age in the time of biting activity [Lumsden (56)]; such a change also occurs in *Simulium* [Davies (22); Lewis (55)]. If the biting cycle is related to an endocrine cycle, a study of ageing effects might be of considerable interest.

Although cycles of flight and biting activity in different species of *Aedes* may show widely differing patterns, the oviposition cycles may be very similar. Gillett & Haddow (31, 32) have found such a similarity in *Aedes africanus* Theobald and *Aedes aegypti* (Linnaeus) and have shown experimentally that the oviposition cycle is not affected by an imposed feeding rhythm. The time of oviposition is related to the time of the beginning of the light period, but light and darkness both seem to play a role in the cycle. Although a normal oviposition cycle is maintained when there are only 4 hr. of light a day, and, indeed, as little as 5 min. of light are sufficient, the cycle is less definite if 20 hr. of light a day are given [Haddow & Gillett (35, 36)]. Biting cycles of the tsetse fly are well-known [Buxton (16)], and, like mosquitoes, the flies show species differences in the time of the biting phase. The amount of activity under various conditions has not been much investigated, perhaps mistakenly. Vanderplank (89) found that *Glossina* was less active during a day following bright moonlight than on one after no moonlight; further investigation of this phenomenon is needed.

The medical importance of biting insects has stimulated investigation of

their rhythms; far less work has been done on other insects. *Drosophila*, however, has been studied in some detail in the wild state. This work is of interest because the insect is not dependent on the presence of a host for its feeding activities; such a dependence may have affected the evolution of the biting rhythms more than has been considered. The main flight activity of *Drosophila* generally takes place in the morning and another peak occurs before sunset [Dobzhansky & Epling (23); Mitchell & Epling (63); Pavan, Dobzhansky & Burla (74); Taylor & Kalmus (87)]. Both temperature and light intensity influence the timing of the activity and the two may alternate in importance. When temperature is high, the time of activity is mainly determined by light intensity, whereas, at low temperatures, temperature fluctuations determine the times of activity [Dyson-Hudson (25)].

A difference between the two sexes in the time of flight has frequently been noticed [Pinchin & Anderson (75)]. The female of the rice-borer moth, for example, is attracted to light at the time of mating, whereas the male is attracted only after mating. Sex differences may also occur in the time of adult emergence, as will be mentioned later. Experimental work has given practically no attention to this aspect. When the separation of the sexes in a catch or in an experiment is neglected, a less distinct activity rhythm may be recorded, or it may be concluded that the insect is bimodal in activity.

Bimodal activity rhythms have been recorded in a number of insects. Williams (93), in his four years of trapping, only found one such insect, a moth, *Pterophorus monodactylis* (Stephans), although some Diptera had a small activity peak at sunrise in addition to a large one at the beginning of darkness. Two peaks of activity have also been observed in the emigration flight of *Aphis fabae* Scopoli [Johnson (51)], of *Simulium venustum* Say [Davies (21)], and in six species of Trichoptera [Corbett & Tjønneland (20)] and in the biting cycles of *Anopheles* [Mattingly (60); Teesdale (88)].

Nearly all species of *Drosophila* show bimodal activity. Taylor & Kalmus (87) have studied this phenomenon in *Drosophila subobscura* Collin, which has apposition eyes, which are not normally associated with crepuscular activity; indeed, the insect cannot mate in the laboratory in the absence of light. There is, however, a shift in spectral sensitivity to the shorter wavelengths when the light intensity is low [Fingerman & Brown (29)], and Taylor & Kalmus suggest that *Drosophila* has become adapted to dry conditions, not by an increased resistance to desiccation, but by this increased visual efficiency in low light intensities, and that this has been followed by a change in time of flight activity.

It is possible that some of the bimodal rhythms described are, in fact, caused by the transients produced by irregular environmental conditions. If double peaks appear in the laboratory when environmental changes occur at "unexpected," times, it would not be surprising if animals living in relatively

unstable field conditions frequently showed a response to changing conditions by double activity peaks. *Periplaneta* shows two peaks of activity under some circumstances and can retain both peaks in constant conditions for a few days. When *A. aegypti* is exposed to only 4 hr. darkness, a double peak of oviposition occurs [Gillett, Haddow & Corbett (33)]. Clearly, a great deal of work is needed on the basic control of these types of rhythm.

*Emergence rhythms.*—Experimental studies on rhythms of adult emergence have already been discussed, and many field observations support the laboratory evidence of their existence. It is perhaps remarkable that these particular rhythms should be so consistent, for, unlike flight or biting activity, the events leading to emergence cover a considerable period of time and involve complex internal changes in many systems. Palmén (69) has reviewed this subject and mention will be made only of work of interest in the general study of rhythms.

Mell (61) recorded the emergence times of 202 species of Chinese Lepidoptera from a total of 5634 individuals and suggested that pupal emergence occurred at a specific time of day, except in phylogenetically old species.

An interesting observation has been made by Park (72) on the emergence of the syrphid fly *Microdon*, which takes place before dawn, although the larva lives in an environment where there is little fluctuation in light intensity.

Palmén (69, 70) studied a number of species of chironomid, which emerge at a time regulated by the change from light to darkness; he concluded that the rhythm was temperature-independent within the ecological temperature range.

A difference in the time of emergence of the two sexes has been recorded in a number of insects. In the chironomid *Allochironomus crassiforceps* Kieffer, the male emerges before the female, the peaks being maintained for at least six days in constant darkness. Caspers (17) similarly found that the male of *Clunio marinus* Haliday emerges about 2 hr. before the female. Earlier emergence of the male is also recorded in *Aedes taeniorhyncus* (Wiedemann) [Nielson & Haeger (67)] and in cecidomyids [Barnes (1)]. Like the sex variation in time of activity, this aspect does not appear to have been studied experimentally.

*Lunar rhythms.*—Although, as far as is known, lunar rhythms are not directly connected with diurnal rhythms, the characteristics of the internal controlling system are so similar to those of the internal clock that some reference to them should be made.

Hartland-Rowe (47) found both a lunar and diurnal rhythm of emergence in the mayfly *Povilla*. Both rhythms persist in continuous darkness. He suggests that the lunar rhythm may be an adaptation to tropical lake conditions, in which there is very little seasonal change, and notes that, of the 94 species

recorded by Caspers (17) as showing lunar rhythms, 10 per cent live within 10° latitude of the equator. Allowing for the small number of zoologists working in this region, these figures give some support for Hartland-Rowe's theory.

Lunar rhythms have also been recorded in the emergence of chaoborids and chironomids [MacDonald (58)] and in the activity of *Glossina* [Vanderplank (89)].

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# THE THEORETICAL AND PRACTICAL STUDY OF NATURAL INSECT POPULATIONS[1]

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The first five volumes of the *Annual Review of Entomology* contained four articles on essentially the same topic as is discussed below. In the same period, many other papers have been published elsewhere on this subject. The authors are far from reaching agreement on general principles, let alone conclusions. It may be that some authors are more interested in proving their opponents wrong than in providing evidence for alternative theories. But fundamentally, apart from the fact that the subject is a very difficult one, disagreement probably arises from the absence of sufficient evidence of the right kind. Laboratory experiments are, in general, inconclusive, since the detailed methods of culturing insects are an all-important part of the population problem and determine the nature of the results obtained. This is not to say that special problems cannot be usefully studied in the laboratory; it is possible, for instance, to show that predatory mites alone are capable, under some conditions, of controlling the fruit tree red spider, *Metatetranychus ulmi* (Koch), a fact that might take many years to establish under field conditions [Collyer (8)].

Most population theories, so far as they are not purely inductive, are based on imperfect field data that are not derived from planned population studies in which all the relevant factors were measured simultaneously. In 20 or 30 years time, when perhaps more of such fundamental studies will be available, we may be able to discuss our theories with more light and less heat. The present article is a review of the difficulties and an attempt to show how some of them can be solved and is not a review of the whole literature.

One of the difficulties in the theoretical discussions of insect population dynamics has been the lack of agreement about the meanings of terms. At least four terms must be agreed on, and I shall give my own definitions or explanations without insisting that they are the only possible ones, though I believe they may be the most convenient.

The term "population" means all those individuals of one species whose lives are sufficiently integrated to have an influence on one another. A population will normally occupy a spatially fairly well-defined area even though the distribution of a species is hardly ever strictly continuous. Although populations so defined will often tend to have somewhat woolly edges, it is only to such populations that one can correctly attribute such features as birth rates and death rates.

[1] The survey of the literature pertaining to this review was concluded in December, 1959.

## Population Equilibria

Populations, particularly under more or less natural conditions, tend to exist in a state of "equilibrium," perhaps [Nicholson (36)] better called "balance." This is a troublesome term about which there has been much disagreement. The equilibrium is only relative to the vast increases in numbers or to the sudden extinction that is theoretically possible. Even occasional fluctuations with an amplitude from peak to trough of several magnitudes can well be accommodated within this definition. The following example will serve to show what is meant by the term.

There have been 68 species of British butterflies; of these, seven are rare migrants, three common migrants (but not overwintering), 28 common, 17 local, 10 very local species, one is almost extinct, and two are extinct. In spite of the very large changes in the vegetation and the general reduction of suitable areas, there has been very little change in status for the last 150 years. The local species have tended to get more local, and, undoubtedly, a number of particular populations have become extinct, usually because of the modification of the habitat, possibly assisted in a very few cases by overcollecting.

Only five or six species seem to call for comment. The large copper, *Lycaena dispar* (Haworth), has been extinct since about 1865; this was almost certainly a result of the draining of the fens. The mazarine blue, *Cyaniris semiargus* (von Rottemberg), has been extinct since 1877; the reasons are far from clear but probably lie in the changes induced in the vegetation of the habitat, which was always very localized. *Aporia crataegi* (Linnaeus), the black-veined white, which is now almost extinct, has probably been partly reduced by the spraying of fruit trees, which has gradually increased in intensity over the last 70 years. The marsh and heath fritillaries, *Euphydryas aurinia* (von Rottemberg) and *Melitaea athalia* (von Rottemberg), have become much more local and rarer; this is probably mainly caused by the destruction of the habitat. In my own lifetime, I have seen one flourishing colony of the first-named species destroyed by building. Finally, the comma butterfly, *Polygonia c-album* (Linnaeus), was widespread a hundred years ago, became very local about 1900, and is now much commoner and more widespread again [Ford (21)];[2] this could be attributed to secular changes in climate, but the causes are not really known. The environmental changes in Britain brought about by human action in the last 100 years are such that under natural conditions they would only happen in hundreds of years.

Thus, the more or less evident equilibrium of about three-quarters of the species is very striking. A species such as the Lulworth skipper, *Thymelicus sylvestris* (Poda), may be specially mentioned. It was found to inhabit a

[2] Andrewartha & Birch (1, p. 664) cite some of the same data but mainly as evidence against the existence of prolonged balance.

small area in Dorset in 1832 and still lives there at about the same degree of abundance. The equilibria are not, of course, everlasting but under natural conditions would often last many years (perhaps hundreds). The size of the colony is obviously important; the smaller ones are more likely to experience catastrophes [see discussion by Skellam (44)].

Dethier (17) describes an interesting example in *Melitaea harrisii* (Scudder), a butterfly with a single food-plant, a species of aster, and little faculty for distributing its eggs according to the amount of the plant available, so that many half-grown larvae die in the search for more food. The plant itself being characteristic of a particular stage in succession is subject to big fluctuations in numbers. As a result, the insect is inefficient at producing adults from eggs. None the less, the populations described by Dethier seem to me (though not to him) to be in a state of approximate balance, with the density of the species determined ultimately, though perhaps at rather long intervals, by competition.

Some insects, such as locusts, give the impression that no equilibrium is ever reached. Equilibrium could in any case be expected only in the outbreak centres [Gunn (27)]. The species that gives the most marked impression of lacking any equilibrium is the desert locust, *Schistocerca gregaria* (Forskål), which has no clearly defined outbreak centre. As pointed out by Nicholson (37, p. 108), in marginal areas there may be a balance between extinction and immigration without any real equilibrium being reached. It is arguable that the desert locust populations as a whole live in such a situation.

## Controlling Processes

Prolonged equilibria are attributable to the action of "controlling processes." The oldest and most hallowed use of the word control means that a pest is reduced to a level at which there is no serious economic damage. It is clearly impossible to forbid this usage, but in population theory something different is meant by control. Living organisms always tend to increase exponentially and can only ultimately be prevented from doing so by processes that are themselves exponential and are in some sense geared to the changes in the numbers of the organism; that is, controlling processes are, in the most general sense, "density-dependent." I doubt personally whether it is useful, at this stage of our knowledge, to try to define density-dependent very closely. I believe that nearly everyone intuitively perceives this truth, which is essentially inductive, and I assume it here as an axiom. Some even of those who appear to dispute it seem to me to give their case away. Thus, Thompson (47, p. 275; 49, p. 382) says, "As [an organism] increases in numbers it necessarily spreads both in space and in time." This clearly implies that the spread into suboptimal habitats, which Thompson thinks is an important controlling agency, is density-dependent. For, if relatively more individuals do not leave the optimal habitat as their density increases,

there will be competition for food or some other necessary requisite.

While controlling processes must be in some sense density-dependent, the reverse is not true. A parasite, for instance, can be dependent on the density of its host but have too low a reproductive rate or too poor a dispersal rate to be able to control it. Utida (50) describes an extraordinary case in which *Callosobruchus chinensis* (Linnaeus) under laboratory conditions maintains a density of 350 in the presence of a chalcid parasite and of only 250 in the absence of the parasite when intraspecific competition is more severe. It is, however, very dangerous to select single factors from the complex in which they act in nature. In particular, insect growth and reproductive rates are so sensitive to temperature, and sometimes also to rainfall, that small changes in climate determine whether a particular agency can or cannot exercise control. Clearly, climate by itself cannot generally act as a controlling agency, because, apart from prolonged secular changes, the incidence of years, which are good or bad climatically, must be random in respect to the present state of the population. In spite of this, in one of the cases (British grasshoppers), which I, myself, have studied in detail [Richards & Waloff (43)], and probably in a great many others, the main factor accounting for changes in numbers from one year to the next is climate. There is no reason, even in theory, why a run of suitable years should not exercise complete control (in any sense of the word), but such runs must, in the nature of things, be relatively short. One of the difficulties of the present state of theory is that we cannot predict in a real world how soon density-dependent processes must intervene.

It would seem that much disagreement has arisen from taking insufficient account of the different periods that elapse between the intervention of various factors and the appearance of visible effects on the insect population. Most mathematical population theories assume that factors have an immediate effect on numbers and usually also that reproduction is continuous. Perhaps this discrepancy between nature and theory is the substantial basis in the proposition of Andrewartha & Birch (1, p. 661) that populations can be controlled by the quantity $r$ being positive for too short a period, that is (essentially), that reproductive increase does not exceed mortality for a long enough period.

Many of the effects of climate on insect populations are almost instantaneous, as when frost, rain, or drought destroy large numbers. So too are some types of competition for food, as for instance in carrion flies. The reduction in the reproductive rate or in the rate of development by unsuitable conditions act rather more slowly, while conditions (e.g., shortage of food) that reduce the size of the females and make them lay fewer eggs take still longer to have an effect. On the other hand, parasites or predators, usually regarded as including the most characteristic density-dependent agents, will act more slowly still, since to have a larger proportional effect at least one generation of reproduction will be required. In most temperate

and many dry tropical countries, species often have only one or two generations a year, so that such biotic effects are likely to be very slow compared with climatic ones [cf. Lack (31, ch. 11)]. For this reason Varley (51, p. 140) has proposed the term "delayed density-dependent factors," but the delay is a matter of degree, partly depending on the climate and on voltinism. Very few influences on the level of population are instantaneous in their effects.

In several experiments [Davidson & Andrewartha (11, 12); Williams (52, 53)], it has been possible by means of multiple regression equations to calculate from the climatic record the expected deviations of insect populations. Such calculations account for 60 per cent or more of the actual deviations, and the remaining error may be regarded as relatively small in a problem concerning living animals in nature. Such regression equations, however, are normally concerned with the recent past and near future; they are scarcely concerned with the longer term equilibrium with which density-dependent factors are concerned. As Nicholson has stated, there is an equilibrium from which the deviations are measured and which the regression does not explain. It was with these considerations in mind that I wrote [Richards (41, p. 86)] that the short-term and long-term population problems were different and that multivariate analysis, though the best method (in general) for studying the former, could not be used to study the latter.

## Importance of Climate

Nothing is better established in zoogeography than the existence of life-zones that differ in their flora and fauna. Part of the differences are attributable to the geological history of the regions, but mostly to the climate of the zones. Moreover, as a species reaches the edge of its area of distribution, it reproduces more slowly and becomes rarer until finally zones are reached where it is an irregular casual [Filipjev (20)]. How can these well-known facts be reconciled with the statement that climate cannot control populations? This I believe is the most fundamental objection raised by Andrewartha & Birch to the more extreme statements of the overriding importance of density-dependent factors. While no one perhaps can give a completely general answer, one can be sought along such lines as the following.

At the extreme edge of the range of a species, chance will play an increasing part in control: immigration and extinction tend to balance, and the density of the species has little importance in control. In the centre of the range, where the climate and other features of the habitat are optimal, "classical" Nicholsonian control will normally operate in most years. Deviation from the conditions of the central area (e.g., in temperature) will usually reduce the reproductive rate, which is generally a very sensitive index of conditions. The species will also tend to have a more patchy distribution because of the need for, say, sheltered situations on south-facing

slopes. While these changes might happen to affect all the causes of mortality equally, so as to compensate for the reduced reproductive rate, this can only be expected to happen rarely; generally with a lower reproductive rate, the species will tend to be rarer. One should distinguish here between short-term ups and downs in the rate and a long-term lower average. Climate by itself can only raise or lower the reproductive rate, leading, potentially, to indefinite multiplication or extinction; a system reponsive to the density of the species is necessary to provide a state of balance. This point is discussed in more detail by Nicholson (36, p. 43) [cf. also Williamson (54)].

It may seem anomalous to state that there can be a regular gradient in the numbers of a species which is directly related to climate and yet to deny that climate controls the numbers. This is partly because of the ambiguity in the word control; in Nicholson's sense, the climate is "legislative" but not "governing." Climate can determine whether a species would multiply indefinitely or would become extinct (though the rates of these processes might vary widely), but only a density-dependent process could produce a prolonged state of balance. While, in practice, control is usually exercised by a complex of factors, we can say that it could not (for all practical purposes) be exercised by climate alone, whereas it could be by a suitable density-dependent agency even if climate were constant.

## Importance of Time Scale

The nature of population problems alters with the time-scale on which we focus. Over long periods, in which geological processes are involved, we are familiar chiefly with vast population changes, either invasions of new areas or extinctions or restrictions to localized refuges. There are analogies to the first alternative in some of the recent artificial introductions [Elton (19)]. The long-term natural restrictions seem to be caused by either secular changes in climate or by the opening of invasion paths, like the route across the English channel, which expose previously isolated species to new competition.

A much more difficult process to substantiate but, nevertheless, one that must have been universally operative over somewhat shorter periods is the establishment, for each species, of population levels that correspond to the local average climate and the relevant parts of the local flora and fauna. This is the process that gives rise to the recognisable life-zones and is certainly partly attributable to natural selection of adapted genotypes and partly to the establishment of a natural state of balance in the sense defined earlier.

## Life Tables

Finally, there are the short-term fluctuations, especially the annual ones, in which climate certainly plays a dominant part, though it often also acts indirectly through biotic agencies. Population dynamics, by definition, deals chiefly with these short-term changes. At the present moment we can

hardly hope to do more than explain things after they have happened, since in any case there is no immediate prospect of predicting the weather a year ahead. Perhaps one of the greatest real sources of controversy at the moment is the division of interest between those for whom the most important or interesting phenomena are those of balance and those who are more interested in explaining fluctuations. The difference in interest is particularly likely to lead to controversy because it is impossible to obtain satisfactory numerical solutions that would enable us to define at all closely what we mean by short- or long-term processes.

If the sexes are produced in equal numbers, it is clear that a species that lays 100 eggs, not a high number for an insect, must have a 98 per cent mortality if the population is to be stable. A deviation of only 0.5 per cent in the mortality will result in a 25 per cent increase or decrease in the population in the next generation. How is this to be reconciled with the observed fact that large variations in such mortality factors as parasitism do not necessarily lead to the expected large alteration in the level of the population? Most of this excess stability in the population can be accounted for by the homoiostatic effect of overlapping causes of death, so that individuals that in any particular year escape one source of mortality merely survive to encounter one of the others. No other arrangement could account for the relative stability of many populations in most years; adjustments by density-dependent factors are usually too slow and in some cases are actually observed to be absent. In general, only a part, sometimes a small part, of the mortality attributable to any one cause is "irreplaceable mortality," in the sense of Thompson (48).

This problem of assessing the relative significance of mortality agents brings us to the importance of the life table. The life table, whether set out formally in the way approved by actuaries [Deevey (13); Morris & Miller (35)] or more simply as an annual budget of a species with numerical estimates of the successive causes of mortality, should be the keystone of all population studies. Logically, in the absence of a complete life table no reliable statements can be made about the importance of any one cause of mortality. No doubt this is a somewhat extreme statement, since by various indirect methods a good many more or less reliable assessments can be made before a life table is available. Nevertheless, it is desirable to keep constantly in mind the danger that in the absence of a life table it is very difficult to assess the importance of any one factor. The example of *Hypera postica* (Gyllenhal) (=*Phytonomus posticus*) in Montana is very instructive. In certain fields, the ichneumonid, *Bathyplectes curculionis* (Thomson), may parasitize as many as 99 per cent of the larvae, but in some years the factor that actually seems to determine the fluctuations of economic importance is the date the alfalfa is cut, which kills almost all the larvae [Reeves & Hamlin (40)].

It should also be added that while a life table is essential to understand

what has happened in any one year, it does not make predictions possible. Adjustments of population levels may take place over several years, and it is usually necessary to have life tables for several consecutive years to assess the permanent value of individual factors. Moreover, in a climate like that of Great Britain, all years are in some respects exceptional, and one requires a five-year period as an absolute minimum to get useful ideas about population dynamics. Finally, as pointed out by Morris (33), the factors that have important effects on the yearly changes in numbers are those that vary; constant factors, however large the mortality they cause, have no dynamic effects and a series of years is required to detect which factors vary.

Attempts to construct life tables of insects are still in a very early stage. A series of examples may be arranged so that the problem becomes increasingly difficult to solve. A brief classification of the problem was given by Richards (42). The most important complications to consider are those that arise from the type of population, whether compact or diffuse, large or small, and from the breeding habits of the species, which may result in a one-stage or multiple-stage population [Taylor (46)] and in discrete or overlapping generations. The simplest examples are those in which there is a topographically well-defined, moderately dense, not too mobile population that occurs throughout most of the year in a one-stage condition and has non-overlapping generations. The various complications of this, the simplest case, involve difficulties both of sampling and of interpretation.

The subject of sampling will not be discussed as it has recently been dealt with by Morris (34), but it is clear that rare species or very mobile diffuse populations will always be difficult to sample. The first analysis of the sampling data should, at least in theory, aim at determining the type of distribution, since this will in turn indicate how to calculate fiducial limits. A random or Poisson type of distribution has been assumed almost universally, though much evidence suggests that distributions showing some aggregation or overdispersion are much more common. According to Bliss & Fisher (4) they can normally be fitted with a negative binomial distribution, and these authors show how to calculate the standard error of the mean in this case. As a species gets rarer, its distribution usually gets less and less distinguishable from the Poisson type.

It is convenient at this point to refer again to the difference between a conventional life table and what may be referred to as a "budget." Life tables were originally devised as a tool for analysing human populations. Their main difficulty is that they do not determine the cause of death but allow for the effects of varying age-structures in populations of a long-lived, slowly multiplying species, which may have a considerable post-reproductive period. Insects are much shorter lived, rarely outlasting a year, and the main problem is to determine the causes of death. In a great many cases, the population is in a one-stage condition a good part of the year, and thus there is very little time for an age-structure to develop. As indicated by Morris

& Miller (35), only a simplified life table is normally useful, and this is what I would call a budget, namely a set of consecutive measurements of the population in which the changes from one occasion to the next have been measured and, as far as possible, accounted for.

### Determination of Age Structure and Mortality

Although insect populations do not have an age-structure of the long-lived vertebrate type, a series of recognisable instars does exist in nearly all species and can serve to break up the population, though the different instars do not necessarily correspond to equal intervals of time. The stages and instars of an insect normally provide the most powerful tool for analysing the seasonal changes in numbers. Moreover, if the relation of instar-length to temperature is determined experimentally, it will be possible to predict the mean lengths of the instars at the prevailing field temperatures by using the formulae of Davidson (10) or perhaps, if the species is specially susceptible to varying conditions, those of Pradhan (38, 39). In this way, the analysis of instar mortalities will correspond to mean intervals of time.

Only in the simplest case do the instars follow one another in a regular seasonal succession. When this happens, as in the spruce budworm, *Choristoneura fumiferana* (Clemens) [Morris & Miller (35)], estimates of the population on successive dates show the course of mortality. This simple condition arises because the eggs are all laid over a period that is very short compared with the total life history, and thereafter the population effectively changes from one instar to the next on the same date. It is much more usual for most of the instars to occur in the field simultaneously. If it is possible to measure the mortalities of each instar, they will not then be consecutive but more or less simultaneous; they are consecutive for the individuals but not for the populations. It is also quite common for individuals to awake from hibernation over a long period or for the egg-laying period to be prolonged. In this case, not only will the different instars occur together but the seasonal increase and decrease in the population will overlap so much that successive measurements of the total population cannot be used to show the course of its diminution.

Two methods of dealing with this problem have lately been devised. One has been tested experimentally under laboratory conditions [Dempster (14)] and also has proved usable for some species in the field [Dempster (15); Richards & Waloff (43)]. The other method has only been tested on the chrysomelid beetle *Phytodecta olivacea* (Forster), on which a paper is in preparation [cf. Richards (42)]. In all such methods the proof of the pudding is in the eating: if they seem capable of giving consistent answers, they are useful, but this depends as much on minor details in the behaviour of the insect as on the skill of the ecologist. The starting point must be a programme of frequent sampling that measures the size of the population and the proportion of the instars in it. The sampling intervals must be short enough

to allow for very rapid growth at some seasons and also for variations in the weather. The intervals must be regular so that each form is sampled in proportion to its abundance and must be prolonged over the whole active season for the same reason. There is often a conflict between the desirable sampling programme and what is physically possible for the entomologist or what the habitat and the species can stand. At the end of the season such sampling allows the drawing of a series of curves that show the abundance and the occurrence in time of the successive instars of the species. The curves will usually largely overlap one another.

If the curves have a single maximum to which they rise rapidly, that is, if the period when recruitment and mortality overlap is short, they can be analysed by the first method, which assumes that the mortality in any one instar is either constant or can be legitimately expressed as an average. On this assumption, the slope of the curve after the peak measures the fall-off, which is caused by the combined effects of mortality and entry into the next instar. The curve can usually be turned into something like a straight line by taking logarithms of the population numbers, and a line can then be fitted by the method of least squares. Extending this straight line back to meet the axis on the date when the instar first appeared in the field gives an estimate of the total number originally recruited into the instar. This can be done to each instar in turn and the differences between the numbers recruited to the successive instars measure the mortality in each instar.

In the chrysomelid beetle *Phytodecta*, oviposition is so prolonged that the curves for the instars overlap greatly and, because of the importance of predation in the destruction of eggs, tend to be flat-topped or even bimodal. In most years there is no distinct peak in the numbers of the instars, and it is impossible to calculate the fall-off in numbers, since recruitment and mortality overlap almost completely. The following method has been used by Dr. Waloff and myself, to analyse these curves. The number of eggs laid weekly in the field is deduced from weekly estimates of the numbers of females combined with regression curves based on experimental data and relating weekly oviposition to mean temperature and the mean age of the population. The total of eggs laid in the season gives the initial population, which is assumed to suffer a constant mortality in each stage, i.e., the actual mortality is averaged separately for any one instar. Normally it will be different for each one, even if they are of the same duration. To estimate the effects of this mean mortality it is necessary to know the duration of each stage, and this will have to be determined experimentally at several temperatures. To take then the egg stage as an example, if $N$ be the total number of eggs found in regular field samples, $n$ the total number of eggs laid in the field, $a$ the duration of the egg stage at the average temperature in the field at the relevant period, and $k$ the average fraction surviving per unit time during the egg stage, $N=\int_0^a nk^t dt = n(k^a-1)/\log_e k$. If $N$, $n$, and $a$ are known, this can be solved for $k$ by trial and error and in the last stage by

linear approximation. The number of eggs surviving or the number of larvae entering the first stage will be $nk^a$, and the same method can be applied to this stage and to the succeeding ones, providing they can all be equally sampled in proportion to their abundance.

### Determination of Agents of Mortality

Such methods only give the total mortality in each instar and do not indicate the causes. They depend on various simplifying assumptions, which may or may not be justifiable, depending partly on the species. It is therefore essential to have as many independent estimates of the figures as possible. This supporting evidence should be of two kinds: independent estimates of particular figures, e.g., estimates of numbers of adults by marking and releasing as well as by counts on quadrats; and measurements of all known causes of mortality to see if they add up to the totals deduced from the instar curves. The latter, of course, give a good deal of help in looking for likely causes of mortality.

The four most likely causes of mortality are weather, parasites, predators, and disease. It should be realized that the effects of weather on rates of reproduction or development, whether direct or indirect, are already included in the formulae.

In most insects, deaths caused by weather are very difficult to demonstrate directly in the field. A large body of somewhat anecdotal evidence is often established, but dead insects are seldom found. Certainly most insects suffer large mortalities during the winter, but how much of this is directly attributable to climatic agencies and how much to indirect effects through fungi, etc., is very uncertain. In general, the most powerful method of investigation seems to be attempts to relate mortality to selected climatic factors by means of correlations or regression equations. Clearly intuition as well as knowledge plays some part in choosing the supposedly important factors. It is, in fact, highly desirable to establish experimentally that effects deduced from regression equations actually occur; for instance, if mortality is shown to be related to the number of days on which the temperature does not exceed $x°$, it is desirable to show also that temperatures of $x°$ in the laboratory do have an adverse effect on the species. Effects of factors such as heavy rainstorms can, in theory, be measured by enumerations of the populations before and afterwards, but, in practice, this is usually difficult to do with sufficient accuracy. An impression gained in Britain is that the direct effects of weather during the summer months are very unimportant compared with the indirect effects on reproduction and development. A very successful use of such regression equations in Africa has recently been published by Gunn & Symmons (28) and Symmons (45). The numbers of red locust, *Nomadacris septemfasciata* (Serville), can be accurately forecast in the outbreak centres from the number in the previous season combined with the previous season's rainfall. The forecasts are for six or more months.

A slightly different expression of climatic effect is indicated in the theory of climatic release. This suggests that normally the climate of a region may not allow the multiplication of a species to prevail against the causes of mortality, but a year or a succession of years of suitable weather allows an outbreak to occur. This was implicit in Cook's work on cutworms (9) and is discussed more explicitly by Greenbank (25) in the spruce budworm.

Parasitism of insects by other insects is usually the best measured of all the causes of mortality. To obtain reliable figures a carefully planned and extensive series of samples have to be dissected. These must cover the whole season and all stages [cf. Miller (32)]. The programme thus very conveniently forms a part of the sampling which is in any case necessary to estimate the changes in the population. Dissection always provides more information than rearing parasites, though the latter is also necessary for purposes of identification.

The measurement of the effects of predation is more difficult and presents problems that differ according to whether vertebrates (usually birds or mammals), insect, or other arthropod predators are considered. No universally applicable solution of these difficulties is available. Vertebrates are generally so rare that the removal of an appreciable sample to study their gut contents would often alter the whole situation. It is often very difficult to recognize a particular species of insect amongst the gut contents. On the other hand, methods of estimating the numbers of vertebrates are well established, and it may be possible by special studies to measure their effects [e.g., Buckner (7); Gibb (24); Holling (29)]. Such studies are likely to differ considerably in each particular example.

In regard to insect predators, serological methods have lately been developed which in favourable cases make possible the specific identification of prey [Brooke & Proske (6); Fox & MacLellan (22)]. Favourable cases are those in which the tissues of the prey species produce suitable antibodies when injected into a rabbit or other vertebrate host. This antibody must be specifically distinct so that the prey can be differentiated from allied species or genera that may occur in the same habitat. Given these advantages, the presence of the tissue of the prey in the insect predator gut can be established. It is still necessary to find out how long such tissues remain in the gut in a recognizable condition and also to measure the size of the populations from which the samples of predators have been taken. The whole process involves a great deal of work, but it has been possible to make quantitative estimates of the predation on the beetle *Phytodecta* [pupa: Dempster, Richards & Waloff (16); eggs and larva: Dempster (in preparation)]. An alternative method available in some species, but which probably involves at least as many technical difficulties, is the radioactive marking of the prey [Baldwin, James & Welch (3)]. However, unless the prey can be easily marked, as with mosquito larvae, this process is probably more laborious.

Still simpler methods, which may yet give a useful measure of the rate

of predation, are described by Franz (23). He describes how aphids on a spruce trunk can be protected by a wire cage treated with a contact insecticide. Their survival can then be compared with that of fully exposed aphids or of aphids covered by a cage with holes to admit predators. As always, each method is applicable only to species with appropriate habits.

Diseases of insects could perhaps be treated merely as a special case of parasitism, but the techniques required for their detection and the methods by which they spread are different. With the exception of the virus diseases, there often appears to be a considerable interaction with the weather, perhaps more markedly so than in the case of most insect parasites. In general, the sort of sampling programme that will measure insect parasitism will also measure disease, at least as soon as the symptoms are sufficiently patent. Balch & Bird (2) describe the detailed assessment of a disease of the spruce sawfly *Gilpinia hercyniae* (Hartig).

A great many species have a dispersal flight at some stage in their life history. Even where this is not spectacular because many individuals fly at once, as in locusts, aphids, or the spruce budworm [Greenbank (26)], it may still result in movement of a large part of the population. Although dispersal flight is ultimately beneficial to the species by allowing it to exploit new sources of food, it must at the same time, as stressed by Thompson (49), lead to many deaths. In locusts, dispersal is certainly density-dependent, and in aphids, where the percentage of winged forms produced depends largely on crowding [Broadbent (5)], it probably is.

The measurement of mortality will be much more difficult in mobile populations unless the movement is sufficiently random to allow rates to be measured locally and extrapolated over a large area. The more usual situation is perhaps one where there are limited, but far from random, movements. This will make it difficult or impossible to define the area to which any measurements of mortality rates apply, so that no budget can be constructed. Only the most rudimentary attempts [Dempster (15); Jackson (30)] have been made to measure movements of populations numerically. Highly mobile populations are probably not yet suitable for detailed population analysis. A compromise adopted by some workers on aphids [e.g., Dunn & Wright (18)] is to treat the production of alate forms which leave the habitat as equivalent to an additional cause of mortality which can be measured. This is certainly a useful device, but it only postpones the full analysis of the situation.

### Use of Life Table Data

The check on the completeness of the life table suggested in the preceding paragraphs is to sample the population regularly throughout the season and to have a continuous record of reproduction and of the mortality factors. This is more elaborate than, but not different in principle from, the "index of population trend" suggested by Balch & Bird (2). They suggest that the

index (population in year $n$/population in year $n+1$) $\times$ 100 should be compared with the total percentage of mortality accounted for in the life table.

As stated earlier, a complete life table tells what happened in a particular year but, as it stands, does not enable prediction of future changes. Apart from the unknown future influence of the weather, parasite and predator populations will alter in ways that depend on their own reproductive rates. Thus any attempt to make detailed deductions from even several consecutive years of life tables will involve study of the biology of the more important parasites and predators in some detail. Not only are their reproductive rates under various conditions important, but their behaviour toward their host or prey at different population levels is, as Nicholson has stressed, usually more important than the percentage parasitism or predation in a particular year.

A typical one-year population study involves the determination of a number of parameters. For instance, in a univoltine holometabolous species hibernating as an adult there are (*a*) an emergence rate in the spring, (*b*) an oviposition rate, (*c*) the durations and mortalities of each preadult stage, and (*d*) adult winter mortality. If there are four larval instars, there will then be at least 15 parameters. It would be theoretically possible to set up 16 simultaneous equations that could be solved to estimate the parameters. The variables, the observed values of which would be substituted in the equations in order to calculate the parameters, would be a series of estimates of the number of each stage and certain climatic data—at least temperature records. It would be a calculation for which one of the large digital computers would be necessary, but there seems to be no reason why this approach should not be made soon.

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# PRINCIPLES OF INSECT PREDATION[1,2]

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The ultimate aim of studies of predation and other population processes is to provide a basis for the understanding of the dynamics of animal populations. Our knowledge of the way predation operates is hardly precise enough as yet to provide a clear picture of the effects of predation on insect populations. But recent work has at least begun to show what must be studied, has suggested the organizational framework upon which ideas must be built, and has explained portions of certain important components of predation. Hence, the aim of this review will be to demonstrate the components of predation, to designate their characteristics insofar as possible, and to provide specific explanations for some of these characteristics.

Since any complex process is comprised of the effects of a number of variables, the explanation of the process requires a description and precise explanation of these effects or components. Such an analysis should be organized to avoid placing emphasis on one or a few components that are actually only a part of the whole process. Until the importance of the components is known, the most useful system of organization is based on the universality of their occurrence. Thus, those components that are always present may be termed "basic," while the remainder, present in some situations and not in others, may be termed "subsidiary" components. The identification of these components as basic or subsidiary can be based on general observation and logical inferences, and, when they have been identified, they can be described and explained. It will then be obvious that some of them, either basic or subsidiary, will be so unimportant in certain situations that they can be ignored. The first obvious step, then, is to describe and explain the basic components, for their explanation will be at least part of the explanation of all examples of predation. Descriptions and explanations of the subsidiary components will then complete the description of any specific example of predation. I have summarized, in Figure 1, the scheme of predation that has developed from this review and have included it here so that the reader might more easily follow the arguments by referring to it periodically.

## The Basic Components

Previous literature on predation convinces one that many factors can affect predation. These factors can be classed into five main groups in a manner similar to that proposed by Leopold (36): (*a*) prey density, (*b*)

[1] The survey of the literature pertaining to this review was concluded in April, 1960.

[2] Contribution No. 640, Forest Biology Division, Research Branch, Department of Agriculture, Ottawa, Canada.

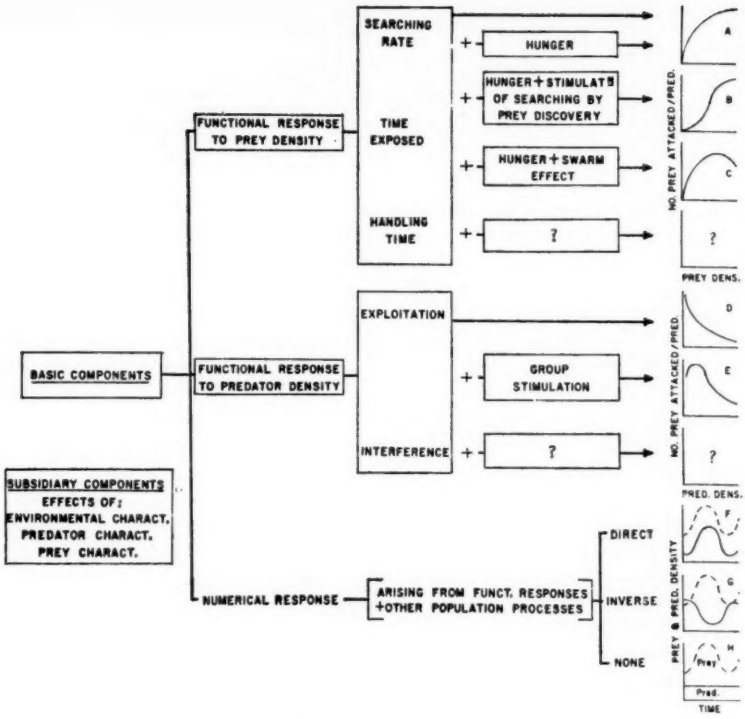


FIG. 1. Structure of predation.

predator density, (*c*) characteristics of the environment (e.g., number and variety of alternate foods), (*d*) characteristics of the prey (e.g., defence mechanisms), and (*e*) characteristics of the predator (e.g., attack techniques). Two of these variables, prey and predator density, are inevitable features of every predator-prey situation, so that the basic components of predation will arise from these universal variables. In at least some situations, the remaining factors are constant or absent [e.g., small mammals attacking sawfly cocoons (27)], so that the subsidiary components will be represented by the effects of the subsidiary variables: environmental characteristics, prey characteristics, and predator characteristics.

*Functional response to prey density.*—The total number of prey destroyed by predators is the product of the number killed per predator and the number of predators that are present. Solomon (48) aptly proposed two terms to describe this twofold nature of predation, the functional response,

concerning prey consumption, and the numerical response, concerning the density of predators.

The published information on the functional response of predators to prey density deals primarily with a special type of predator, i.e., insect parasites. Burnett's series of experiments (9,11), in which fixed numbers of *Dahlbominus fuscipennis* (Zetterstedt) searched for sawfly cocoons at various densities, are particularly well-documented examples of functional responses. In every instance, the parasites attacked more hosts as host density increased. The relationship was curvilinear, however, for the slope of the curves decreased until the curves levelled (Figure 1, curve A). These functional response curves are qualitatively similar to those of *Chelonus texanus* Cresson searching for eggs of *Anagasta kühniella* (Zeller) (54), of *Cryptus inornatus* Pratt searching for cocoons of the beet webworm *Loxostege sticticalis* (Linnaeus) (55), and of *Nasonia vitripennis* (Walker) searching for puparia of *Musca domestica* Linnaeus (15). The most convincing demonstrations of the functional response of parasites operating under completely natural conditions have been presented by Miller (38, 39) for the parasites *Apanteles fumiferanae* Viereck and *Glypta fumiferanae* (Viereck) attacking the spruce budworm *Choristoneura fumiferana* (Clemens). Again, the functional response curves of these parasites rose as prey density rose, but the slope gradually decreased until the curves levelled.

Such functional responses are not restricted to parasites. In laboratory experiments (unpublished data), an unidentified species of corixid and the dytiscid *Acilius semisulcatus* Aubé responded to densities of mosquito larvae in the same way that parasites respond to densities of their host. A decreasing slope also characterized the functional response curves of the belostomatid *Lethocerus* sp. attacking tadpoles.

The functional response of vertebrate predators to density of their insect prey appears to be of a different kind. Three species of small mammals, the deermouse, the short-tailed shrew, and the cinereous shrew, are important predators of cocoons of the European pine sawfly, *Neodiprion sertifer* (Geoffroy), in the plantations of southwestern Ontario (27). In the laboratory and in nature, individuals of each species attacked more prey as prey density increased, but, unlike the responses of insect parasites, the rising phase of each curve had an S-shape (Figure 1, curve B). Leopold (36) predicted the same type of response when he suggested that vertebrate predators attack scarce prey by chance but develop the ability to find a greater proportion where the prey become abundant. Hence, to produce a response curve with an increasing slope, the aggregate number of prey attacked per predator must increase as prey density rises. The limitations imposed by caloric requirements, however, would ultimately cause the curve to level, producing an S-shaped functional response curve. Similarily, Tinbergen (52, 53) proposed that birds preying upon a variety of insects developed a "searching image" of the prey when the prey became dense

enough to make this profitable. Thus, the functional response curves of vertebrate predators in general seem to have an S-shaped rise to a plateau.

A number of factors determine the characteristics of these functional responses. The time predator and prey are exposed and the rate of searching of the predator obviously will influence the magnitude and character of the functional response. Less obvious, perhaps, is the time spent in handling prey; this affects the response by decreasing the time available for active searching. Hunger, too, will play a part and certainly would seem to explain the levelling of the functional responses of small mammals (27). Since so many factors can affect the responses, a basically simple response, which included only those basic factors always present, was analyzed in an earlier paper (28). The data were obtained from an artificial attack situation in which a blindfolded subject—the "predator"—searched for sandpaper discs —the "prey"—tacked to a table at different densities. The only factors affecting the response were the time "predator" and "prey" were exposed, the rate of searching, and the time spent handling discs after they were discovered, features common to every attack situation. The functional response curves obtained from this experiment were similar to those of insect parasites and insect predators, for the number of discs removed from the table increased at a decreasing rate until the curves levelled.

The simplest expression of such a response would be

$$N_A = aT_eN_o \qquad 1.$$

where $N_A$ is the number of discs removed, $N_o$ is the density of discs, $T_e$ is the time available for searching, and $a$ is a constant, the instantaneous rate of discovery. If a fixed interval of time, $T_t$, is allowed for one experiment, $T_e$ must vary with the number of discs found, for the time taken to remove discs decreases the searching time. Thus, if $b$ equals the time to pick up one disc, then

$$T_e = T_t - bN_A. \qquad 2.$$

Substituting Equation 2 in 1:

$$N_A = \frac{T_t a N_o}{1 + abN_o}. \qquad 3.$$

Equation 3 accurately described the functional response to disc density. It also incorporated the correct explanation, since the values for the instantaneous rate of discovery and the time spent in picking up each disc were of the order of magnitude predicted by the equation and were constant at all "prey" densities. Since this equation incorporates only those factors that are basic, it was termed the "basic functional response equation."

The equation was designed only as a first step toward explaining real functional responses. Surprisingly, however, it accurately described all those responses of insect parasites for which data had been published (28), and, subsequently, the responses of three insect predator-prey situations involving pond organisms (unpublished data). In at least one of these

situations (the belostomatid *Lethocerus* sp. attacking tadpoles), the equation incorporated not only a description, but an accurate and complete explanation as well, for only the three basic components operated. In this case, the predator captured and killed even when completely satiated and even when it was feeding. Hence, the functional response rose over enormous ranges of prey densities and levelled only when the identification and strike time comprised all the available time. It is likely, however, that the other situations are more complicated, since hunger and egg complement probably affect the responses. Since the equation describes all these curves so accurately, an accurate explanation of the effects of hunger might have been unintentionally included. If this is true, then an additional time-consuming behaviour must be added to the time spent handling ($b$). This would represent a constant delay in searching as each prey consumed was digested. It is more likely, however, that hunger exerts its effect differently and that the descriptive power of the equation results because it roughly mimics these effects.

Mathematical expressions of biological events are most useful if the parameters have concrete biological meaning. The time of exposure, $T_t$, and time spent handling each prey, $b$, are tangible biological quantities, while the instantaneous rate of discovery is a function of the number of contacts between predator and prey. Stanley (50) developed an equation for the probable number of contacts between a moving particle (*Tribolium* adults or larvae) and a stationary one (*Tribolium* eggs) in a limited volume of flour. This equation was modified by Laing (35) to apply to the parasite *Trichogramma* searching for eggs of the grain moth on a two-dimensional surface and can be expressed as an instantaneous rate as follows:

$$C = V(d' + d'') \qquad 4.$$

where $C$ represents the instantaneous rate of contact (i.e., the area covered), $V$ the mean speed of the predator, and $d'$ and $d''$ the effective diameter of the predator and prey, respectively, so that $(d'+d'')$ equals the width of the area of perception of the predator for the prey. If the prey, as well as the predators, are moving, the equation can be readily modified along the lines suggested by Mosimann's intriguing study (41) of animals searching for mates.

Equation 4 is only part of the instantaneous rate of discovery. Nicholson (42) has pointed out that the number of prey consumed is also related to the ratio of successful prey captures to prey contacts, designated as $S$. Thus, the instantaneous rate of discovery can be expressed as

$$a = V(d' + d'')S \qquad 5.$$

Laing (35) and Fleschner (21) have shown that the terms of this equation can be readily measured in specific predator-prey situations to give a theoretical value that corresponds to the number of prey attacked by actual predators.

The basic functional response equation incorporates an explanation of the effects of the three ubiquitous components: time predator and prey are exposed; discovery rate; and the time spent in identifying, chasing, killing, and eating the prey. There are obviously other, subsidiary, factors that could also affect the functional response in certain situations. The one that first comes to mind is satiation, for the hungrier a predator is, presumably the harder it searches for prey. This, however, is not a universal characteristic of predators, for some continue to attack and kill, even when satiated [e.g., the belostomatid *Lethocerus* sp. (unpublished data)].

Two recent studies of the reaction of blow flies to sugar solutions conducted by Dethier and collaborators (18, 20) have shown how hunger could exert its effect. In one experiment, for example, groups of 100 flies were starved for 24 hours and then fed a 2.0 *M* solution of D-glucose. The degree of hunger was then measured at intervals after the feeding by determining the minimum strength of stimulus, as measured by the concentration of sugar solution, that would just produce a response (extension of proboscis) when applied to the tarsal receptors. Immediately after feeding this "acceptance threshold" was high, but, as time passed, the threshold dropped to an asymptotic value slightly above zero. If only one moderate concentration of sugar solution had been offered at intervals after feeding, there would not have been a response until the acceptance threshold had declined to this value. If the blow fly was not fed, this strength of stimulus would thereafter always elicit a response and, as the acceptance threshold dropped still further, would probably elicit a progressively stronger one. This is just the situation that a predator attacking a particular prey would experience, for each prey would present only one strength of stimulus. If the density of prey was high enough to permit the predator to capture all it required, the acceptance threshold would be so high after each prey was consumed that the strength of stimulus of the prey would be insufficient to release a response. There would be a delay until the threshold dropped. The amount of delay would be related to the level to which the acceptance threshold was raised after each prey was consumed, and this, in turn, would obviously be related to the number of prey eaten during a given period of time. Such an effect can readily be incorporated into the basic functional response equation by adding to the time spent handling ($b$) some function of the number of prey eaten ($N_A$) that describes this "satiation delay." If predators become more responsive to the stimulus of prey as the acceptance threshold continues to drop, the instantaneous rate of discovery might similarily be expressed as some function of $N_A$. The resulting functional response equation will be quite complex.

Gause (22) and Watt (61), however, have deduced a simple and quite different expression to include the effects of hunger. They assumed that a predator can generate a certain maximum number of attacks, $K$, and that the rate of attack is proportional "to still unutilized opportunity" (22) for attack; that is

$$\frac{dN_A}{dN_o} = a(K - N_A), \qquad 6.$$

which integrates to

$$N_A = K(1 - e^{-aN_o}) \qquad 7.$$

Watt (61) showed that this equation accurately described those functional responses of parasites to host density that were discussed earlier. The basic functional response equation similarily described these responses even though the two equations are quite different. Since it was shown in another paper (28) that Watt's equation definitely does not incorporate the correct explanation of the effect of the three basic components, his expression might well explain the effects of hunger. If it does accurately explain these effects, then, where hunger operates, the complete expression must be a combination of the basic functional response equation and Watt's equation. In view of the complex action of hunger, however, the equation probably represents an approximate description of the effects of a number of components, including hunger and time spent handling prey, that exert roughly similar effects upon the number of prey attacked.

The three basic components of the functional response to prey density, alone, or together with the effects of hunger or egg complement, are sufficient to explain the responses of insect parasites and insect predators. Another, fifth, component, however, is necessary to explain the S-shaped functional responses of vertebrate predators. De Ruiter's experiments (17) suggest that birds develop more complex searching patterns than insects. These experiments were designed to test the effect of camouflage by twiglike geometrid caterpillars upon predation by jays, *Garrulus glandarius* Hartert. Hand-raised jays were first exposed, in control experiments, to twigs, which the caterpillars specifically resembled. There was an initial novelty reaction, but, within 24 hours, most birds lost all interest in the sticks. When freshly killed caterpillars were then placed among the sticks, the jays took a very long time to find the first prey. After finding the first larva, however, the birds immediately began to pick up sticks and caterpillars indiscriminantly—a very striking change after the complete absence of interest in sticks demonstrated in the control experiments. The discovery of a palatable prey had stimulated the birds to search specifically for similar objects. If no reward was forthcoming, however, the stimulation disappeared with time. The effect of this stimulation upon the number of prey attacked, therefore, would be related to the frequency of contact, which, in turn, is related to prey density. At low densities of prey, the stimulation received when a prey was discovered would be largely dissipated by the time another prey was contacted, but, as prey density increased, contacts would occur at shorter and shorter intervals before the stimulation had completely disappeared. This effect would produce a functional response curve with an initially increasing slope, and, as the predators became satiated and as more and more time was spent handling prey, the slope would decrease to produce

an S-shaped curve. This fifth component will exert its effect through the instantaneous rate of discovery ($a$ in Equation 3), probably by affecting the ratio of successful prey captures to prey contacts ($S$ in Equation 5). This ratio would thus be a measure of the general responsiveness of predators to the prey and would be expressed as some function of $N_A$.

The two types of functional response to prey density therefore can be explained by combinations of the five components: time predator and prey are exposed, searching rate, handling time (including identification, capture, and consumption), hunger, and stimulation of predator by each prey discovered. The first three are universally present and hence basic and, by themselves or in conjunction with the effects of hunger, can explain those response curves that rise with a continually decreasing slope to a plateau. If stimulation by prey discovery is added to these four components, an S-shaped response results.

Although these five components are sufficient to explain the functional responses that have been reported in the literature, another component that must surely affect some situations, has been examined by Welty [in Allee (2, pp. 98–100)]. He observed that goldfish consumed fewer *Daphnia* when the prey were present as a dense swarm than if they were less dense. Welty attributed this decline to a confusion effect, presuming that several *Daphnia* within the immediate field of vision of the predator offered conflicting stimuli that blocked the feeding response. Dr. R. F. Morris (personal communication) observed a similar effect when the pentatomid predator *Podisus maculiventris* (Say) attacked fall-webworm larvae, *Hyphantria cunea* (Drury), and he attributed it to the continual disturbance of the rather timid predator by large numbers of active prey. Sensory adaptation of receptors as a result of continual stimulation might also be involved. If this sixth and final component does operate, then a third type of functional response—a domed curve—might be expected (Figure 1, curve C).

*Functional response to predator density.*—Predator density, the other basic variable of predation, can also affect the number of prey attacked. De Bach & Smith (16), for example, measured the number of puparia of the house fly, *M. domestica*, attacked by different densities of the parasite *N. vitripennis* and found that the number attacked per parasite decreased as the density of parasites increased, the rate of decrease being most rapid at low prey densities (Figure 1, curve D). Similar curves have been obtained for three other species of parasite, a chalcid (13), a braconid (54), and an ichneumonid (55). Since these data were obtained in laboratory experiments, their reality in natural situations might be questioned. Recently Miller, however, demonstrated similar response for the parasites *A. fumiferanae* (38) and *G. fumiferanae* (39) in the spruce forests of New Brunswick. These studies are not only notable as demonstrations of natural responses to parasite density but also as examples of how field data, if sufficiently accurate and precise, can be analyzed to show meaningful and consistent relationships.

A more complex response was discovered by Burnett for the parasite *D. fuscipennis* searching for cocoons of the European pine sawfly, *N. sertifer*, under laboratory (10) and natural (12) conditions. The number of hosts attacked per female initially increased as parasite density rose and then decreased in the typical manner shown by other parasites (Figure 1, curve E). Although this is the only published example available of an initial increase in the response, the careful design of the experiments convinces the reader that it is a very real feature.

The form of the two types of response curves can be explained by using the same type of component analysis that was used in the previous section to analyze the functional responses to prey density. De Bach & Smith (16) and Ullyett (54, 55) felt that the responses they observed resulted from the action of two components—exploitation and interference. The exploitation component arises because the parasites compete for the same resource. Thus, as the density of parasites increases, the chance that any individual parasite will discover a host that has not already been discovered decreases. Coupled with this is an interference component that becomes progressively more important as parasite density rises and contacts become more frequent. Both these components of the functional response to predator density are probably universal and, hence, basic, for all individual predators compete with other individuals for the same resource and probably interfere with one another in so doing.

The exploitation component forms the basis for Nicholson's well-known model of predator-prey interactions. This model (45) is based on the assumption that the rate of attack is proportional to the density of undiscovered prey. That is,

$$\frac{dN_A}{dP} = a(N_o - N_A),$$

which integrates to

$$N_A = N_o(1 - e^{-aP}) \qquad 9.$$

where $N_A$ is the total number of prey attacked, $N_o$ the original density of prey, $a$ the area of discovery (a constant), and $P$ the density of predators. This equation can be converted to indicate the number of prey attacked per predator by dividing by $P$. Nicholson & Bailey expressed their formulations differently, but they can be readily converted to Equation 9 above.

A number of authors have tested the validity of this expression either by determining how closely it describes attack (16) or by testing data describing functional responses to determine if the area of discovery is a constant, as the model requires (58). The latter approach is more convincing and clearly shows that the area of discovery is not constant. Since a variety of components, of which exploitation is only one, affect these responses, this indicates only that Equation 8 does not contain the complete explanation of attack, a conclusion that is abundantly apparent from the earlier discus-

sions. The validity of this explanation can only be assessed by testing data that are affected by exploitation alone and not by a large number of components. If this expression of the effects of exploitation proves as reasonable as it seems, it could be combined with the basic functional response equation as a first step toward including the effects of predator density.

Interference is another basic component of predator density, and its effects must be included in any realistic analysis. Ullyett observed (55, p. 296) that contacts between females "take up a certain proportion of the experimental period which might otherwise be more profitably employed." This immediately suggests the arguments that were proposed earlier to explain effects of time spent handling prey and indicates that effects of interference can be readily incorporated in the basic functional response equation by subtracting, from the total time ($T_t$), another time-consuming behaviour that is a function of predator density.

Watt (61) has dealt with the effects of both exploitation and interference in his deductive model of predator-prey interaction. Letting $A$ equal the number of prey attacked per predator and using the notation as in Equation 9 above, he stated that "the larger $A$ is, the greater $dA/dP$ will be, because it will be more difficult for parasites or predators to find unattacked hosts or prey." This statement concerns the exploitation component. To include the effects of interference, he added that $dA/dP$ must also decrease in inverse ratio to $P$, "because the greater $P$ is, the greater inter-attack competition will be." These ideas were then specifically expressed in the equation

$$\frac{dA}{dP} = -\frac{iA}{P}, \qquad 10.$$

which integrates to

$$A = aP^{-i} \qquad 11.$$

where $a$ and $i$ are positive constants. When this equation was combined with the effects of prey density discussed earlier (Equation 7), it accurately described a remarkably wide variety of situations (38, 39, 61). Descriptive power alone does not prove the accuracy of the explanation incorporated in an equation, however, particularly in view of Hald's (24, pp. 564–70) demonstration that seven different hypotheses gave statistically acceptable fits to a simple set of data. Final proof must be obtained from experiments designed to test independently the assumptions involved. Nicholson's expression of the effects of exploitation discussed earlier certainly seems more satisfactory, for it is more reasonable to suppose that attack is related to the number of prey remaining than to the number attacked; however, Watt's complete model is the most realistic one available to describe attack. It accurately describes the simpler types of responses to both prey and predator density, and, even if the explanation incorporated is only a rough approximation, it admirably fulfils the descriptive and predictive functions required of a model.

The two components, exploitation and interference, adequately explain those response curves the slopes of which decrease continuously as predator density rises. They are not sufficient, however, to completely explain those curves that initially rise and then decline. An additional component is required that causes individual predators to attack more prey when other predators are present. Now Welty (63) has observed that fish rise to food particles placed on the water surface when they observe another fish rising. This enables a predator to increase its area of perception by responding to another predator that is close enough to a prey to identify it. Since the instantaneous rate of discovery ($a$ in the basic functional response Equation 3) is affected by the width of the field of perception ($d'+d''$ in Equation 4), this group stimulation effect can be incorporated by replacing the constant $a$ with some function of predator density.

As a result of this effect, the number of prey killed per predator will increase as predator density rises, particularly if the prey are aggregated. Thus, even if the first predator removes one prey, there will be others left for the predators following. Defence mechanisms or escape responses of a prey might also be so effective that a single predator could rarely capture it but two or more could. Hence, another portion of the instantaneous rate of searching, the ratio of successful captures to contacts ($S$ in Equation 4), might be directly related to predator density. Finally, Katz's (31, pp. 117–18) comment that hens, apparently fed to repletion as individuals, will start to eat again if they observe another hen eating suggests that the hunger component discussed earlier might also be related to predator density. These behaviours are sufficient to cause the number of prey consumed per predator to rise as predator density increases, but, as exploitation and interference effects become important, at higher densities the curve will decline to produce one resembling the response shown by *D. fuscipennis*.

The functional response to predator density therefore can be affected by at least three components. The simplest situation obtains when only the basic components, exploitation and interference, are operating, and, in such cases, the attack per predator declines as predator density increases. When effects of group stimulation are added, there will be a tendency for an initial rise and then a decline in attack rate. When more complete information is available, these effects can be incorporated, perhaps in the ways suggested, into the basic functional response equation to provide a complete expression of the attack by predators.

*Numerical response.*—To this point, the two basic variables, prey and predator density, have been considered as affecting exclusively the number of prey attacked. This gives rise to two basic components of predation, i.e., the functional response to prey density and the functional response to predator density. Both variables, however, can affect the number of predators present, so that there may be a numerical response in addition to the functional ones.

There are a number of examples where the density of invertebrate preda-

tors in nature increases, apparently, in response to increase in the density of their prey. Huffaker & Kennett's data (30), show that the density of the predatory mites, *Typhlodromus cucumeris* Oudemans and *Typhlodromus reticulatus* Oudemans, increased whenever the density of their prey, the cyclamen mite, *Steneotarsonemus pallidus* (Banks), increased. Similarly a number of predators of the white-fir woolly aphid, *Dreyfusia piceae* (Ratzeburg), also show direct numerical responses; moreover, the number of species of predators present seems to be similarly related to aphid density (46). When prey density is low, the trombidiid mites are the only predators consistently present, whereas, at higher concentrations of prey, the coccinellid *Pullus impexus* Mulsant appear. For the appearance of other species of predator, a mass outbreak of aphid seems necessary. While such changes can be attributed to the delay in dispersal rates of predators into recently infested areas, it seems logical that there are also threshold effects to the numerical responses of predators attributable ultimately to their functional responses, which, below certain prey densities, are so low that the food consumed is not sufficient to permit reproduction or survival.

The densities of many vertebrate predators are also affected by the density of their prey. Certain insectivorous warblers, the Cape May, the bay-breasted, and Tennessee, are consistently more common during outbreaks of the spruce budworm (25, 32, 40). During budworm outbreaks, however, most insectivorous birds show no numerical response to increasing prey density, and some even show a decrease, e.g., the magnolia, myrtle, and black-throated green warblers. Since both Kendeigh (32) and Morris *et al.* (40) observed that the same species of warblers demonstrated the same type of response—direct, inverse, or none (Figure 1, curves F, G, and H)—in different areas and at different times, it is unlikely that they were fortuitous.

Small mammal predators of insects show similar responses. When the European pine sawfly, *N. sertifer*, is abundant in pine plantations, the densities of the cinereous shrew and deer mouse are also high (27). One other species of insectivorous small mammals, the short-tailed shrew, was also commonly present in these plantations, but, because of cover limitations, populations showed no numerical response.

Most of the evidence for numerical responses is based on correlation between predator and prey numbers and is subject to the criticism that the relations are fortuitous. Lack (34), however, has effectively used additional, indirect evidence to show that food limits a variety of vertebrate populations. The most convincing proof, however, would come when an experimenter could manipulate food resources so that a numerical response would always result. Bendell (5) was able to do just this by introducing white-footed mice to a small island before and after an excess of food (wheat grains) was distributed evenly over the island. An introduction of 64 mice to an island lacking a natural population failed when no food was added. A second introduction of 20 mice was then made to the island after an excess

supply of food was added. This addition of food maintained the population of mice where none existed before and where none would exist when introduced without food. Moreover, the densities that were eventually attained were considerably higher than on the control island and the mainland where no food was added. This study thus provides a particularly clear demonstration of a direct numerical response to food.

These examples suggest that there are two possible numerical responses to an increase in prey density, i.e., a direct and an inverse response. When processes other than food limit predator numbers, however, or when the prey are present for only a brief period, some predators will show no response. The inverse relationship has generally been attributed to interspecific competition with more aggressive species, while the more common direct numerical response is usually considered a function of prey density. Actually, the proximate cause is, in part, the amount of food consumed, and this, of course, is reflected by the functional responses to both predator and prey densities.

Previous studies of predation have been concentrated almost exclusively on the direct numerical response. Lack (34) and Andrewartha & Birch (3) have reviewed the effects of food consumption to show that survival, fecundity, and dispersal are related to the amount of food consumed, the first two directly and the last inversely. Hence, at high prey densities when food consumption is high, a greater proportion of predators will survive, they will disperse less, complete their development sooner, and will produce adults that are more fecund. The result will be a higher density of predators. Such an increase, however, is limited. The interference component of the functional response to predator density, discussed earlier, will be one factor, intrinsic to the attack process, that will limit the increase. Other biotic and physical factors that will also affect the response have been analyzed in the reviews of population dynamics that have previously appeared in this publication [Thompson (51); Solomon (49); Nicholson (44); Andrewartha & Birch (4)].

The amount of food consumed is not the only factor arising from the functional responses that can affect the numerical response. Solomon (48) has pointed out that a functional response implies that a predator can attack a fixed number of prey in a shorter interval of time at higher prey densities. Hence, as prey densities increase, more and more time becomes available for other activities, e.g., reproductive, that would promote a direct numerical response.

One final feature of the numerical response deserves comment. Predator density does not respond immediately to a change of prey density, since some time always elapses between the event that leads to the birth of an animal and the appearance of that animal as an active predator. Nicholson (43) argues cogently that this delay will tend to produce oscillations of predator and prey which increase in amplitude with time. Such systems of

growing oscillation are inevitably self-destructive, and, indeed, the mathematical model he developed (45), which incorporated a delay with very specific attributes of the predator, produced such instability. And yet in nature, the self-destructive tendency arising from the delay is somehow avoided, for predator-prey coactions persist. Nicholson's explanation of this apparent disparity involves the observation that insects in nature are not uniformly distributed but are present in groups.

> At any moment some groups of individuals are growing unchecked while others are in process by extinction by parasites, and migration of both hosts and parasites into unoccupied areas takes place all the time, so giving rise to new centres of oscillations [43, p. 42].

The observation that animals occur in groups, however, is hardly proof for this explanation. A variety of factors, e.g., topographical and behavioural, would produce the same fragmentation. Some experimental substantiation has been presented by Huffaker (29), however, when he showed that the interaction between a phytophagous and a predatory mite persisted longer when the experimental universe was sufficiently complex to promote a degree of isolation between the groups.

While such an explanation might explain the sustained equilibrium attained by relatively inactive predators and prey, it hardly seems sufficient to explain the stability of the interaction between highly mobile ones. This has initiated a search by a number of workers for mechanisms that could damp the oscillations. Three principal mechanisms have been proposed (33, 43, 57, 59): (*a*) a density-dependent factor such as food limitation acting on the prey; (*b*) a density-dependent factor acting on the predator so that at high predator densities the predator is less efficient, and (*c*) refuges that will protect a proportion of the prey. Now these mechanisms are usually considered to act upon the predator-prey system from outside the system, a tendency arising from a tacit assumption that Nicholson's model is complete. Since Nicholson himself has said that the attributes he assigned to the predators were simply first approximations, it is worth-while to see if the earlier analysis of the basic responses reveals any features that might act as damping mechanisms intrinsic to the predator-prey system.

In Nicholson's model, the predators are insatiable and the number of prey attacked is directly proportional to the number of contacts. Hence, the functional response to prey density is linear and rises indefinitely with increase in prey density; however, any realistic functional response must level. Subsistence requirements will fix the ultimate level for most predators, but even those that continue attacking after being satiated must have an upper limit that is determined by the time required to handle prey. Two types of curves have already been demonstrated: a curve that rises with a continually decreasing slope and an S-shaped curve. Such responses are immediate. In the first type of response, however, the percentage of predation will decline as prey density rises, and this feature, rather than damping oscillations, will

make them more violent. On the other hand, in the second type of response, the predation percentage will initially rise as prey density increases and then will decline beyond the point of inflection. Thus, if the density of a prey is low, increases in its numbers will immediately increase the percentage of predation. This will tend to limit the increase, and, as prey density declines, the effect of predation will immediately be relaxed. Thus, incipient oscillations can be damped by a mechanism that is intrinsic to the attack process.

Nicholson's predators also have a functional response to predator density, but, as was shown earlier, this involves only the exploitation component. The interference component, which must surely be basic to all situations, is not included. Since the effect of interference is directly related to predator density, the efficiency of individual predators will decline at high predator densities. The resulting relaxation of attack will tend to prevent the prey from being driven to extinction. Hence interference provides another intrinsic damping mechanism.

*Total response.*—Of the five groups of varying factors that affect predation, prey and predator density are the basic ones, for they are universally present. Hence, the three responses to these variables—the functional response to prey density, the functional response to predator density, and the numerical response—are the basic components of predation. In the many situations where the subsidiary factors are constant, predation can be completely described by combining these three components. This has already been done in an earlier paper (27) by assuming that the action of the numerical responses is immediate. While this is, of course, unrealistic, certain general conclusions can justifiably be drawn. The first is that predation tends to be of two types—one in which the percentage of predation declines continuously as prey density rises and one in which the predation percentage rises initially and then declines. The concurrent density-dependent portion of this latter domed curve is produced by the S-shaped functional response to prey density, by the group stimulation effect upon the functional response to predator density, and by the direct numerical response. In such cases, predation would regulate the numbers of an animal whenever the concurrent density-dependent limb of the curve rose high enough to just equal the effective birth rate of the prey. When the prey are insects, their reproductive rate might often be too high for predation alone to regulate. But, if other mortality occurs, density-dependent or density-independent, the total mortality could rise to the point where predators were contributing, completely or partially, to the regulation of the insect.

If the prey happened to achieve the higher densities at which predation was inversely density-dependent, predation would become less effective as prey density rose, and the prey could escape the regulating effect until the populations collapsed and densities returned to the range where mortality from predators was concurrently density-dependent. The possibility for this escape becomes less when the domed predation curve is very broad so that the descending limb is displaced to high prey densities. Since the curve for

each species of predator peaks at a different prey density because of species differences in the basic responses, the more species of predators there are, the broader will be the domed curve of total predation. Hence predator-prey interactions would be most stable in variable environments that harbour a large number of different species of predator. This probably provides at least part of the explanation for Voûte's (60) observation that insect outbreaks are rarer in mixed stands of trees than in pure stands.

### Subsidiary Components

Factors other than prey and predator density can affect predation. Characteristics of the environment, of the predators, and of the prey represent three groups of subsidiary factors that are constant in some situations and variable in others. The effect of these factors are, hence, subsidiary components and, in general, seem to affect the basic responses by changing their magnitude rather than their form.

Burnett (9, 11), for example, found that the functional responses of the parasite *D. fuscipennis* to densities of sawfly cocoons were quite different at different temperatures. Despite these differences, the basic functional response equation described them accurately, the differences simply being reflected by the values assigned to the constants (28). Thus, the instantaneous rate of discovery increased as the temperature increased, while the time spent in non-searching activities decreased. Other attributes of the environment have similar effects. Increase in the palatability or number of alternate foods, for example, decreases the functional response of small mammals to densities of sawfly cocoons (27). The characteristic S-shape is retained however, suggesting that the number and quality of alternate foods simply affects the parameter values.

Differences in the responses of various species of predators reflect differences in predator characteristics, such as their ability to detect, capture, and kill. Fleschner (21), for example, showed that the marked variation in the ability of three species of predatory mites to capture prey after a contact was made was largely attributable to anatomical differences of the grasping mouthparts. These predator characteristics will affect the functional responses through the ratio of number of captures to number of contacts ($S$ in Equation 5). The same ratio will also be affected by the speed of attack of the predator. As Roeder has shown (47), the importance of these times is not reflected by their magnitude, but by the relative times of attack by predators as compared with times of startle by prey. In Roeder's words: "A millisecond or so within the nervous system must often mark the difference between the quick and the dead" (47, p. 290).

The amount of predation will also be affected by the kind of sensory receptor that the predator uses to locate and identify prey. In the artificial predator-prey experiments discussed earlier, the use of different sensory receptors to locate "prey" had a marked effect on the functional response to "prey" density (28). In one experiment, the sense of touch was used to

locate discs, and in another the sense of hearing. Although the latter functional response curve was much lower, both were accurately described by the basic functional response equation when different values were assigned to the constants. Further analysis showed that the sense of hearing decreased the instantaneous rate of discovery by lowering the ratio of successful captures to contacts. This sense also increased the time spent in non-searching activities, for a longer period of time was spent in identifying each disc. Thus, the magnitude, but not the form, of the response was affected by the kind of receptor used.

The type of distribution of prey is one example of a prey characteristic that affects predation. A particularly dramatic example of this effect was presented to Gibb (23) when he observed that tits, preying upon larvae of a cone insect, concentrated their attack in those areas where the larvae were most abundant. This "intensity-dependent" action was so pronounced that a highly variable larval population was reduced to a remarkably uniform one. By responding to the higher densities present in local concentrations rather than to the average density present in their area of search, the functional responses of such predators would be greater.

Contagion occurs to a high degree in such colonial insects as larval *Neodiprion* sawfly, and, if the increased vulnerability arising from such behaviour is so important, it seems paradoxical that colonial behaviour would evolve. There are, of course, survival advantages to colonial behaviour unrelated to predation, e.g., exploitation of resources by groups of individuals. But this solution to such resource problems presents the considerable problem arising from increased predation. It has apparently been solved by the *Neodiprion* sawflies, however, for birds (52) and small mammals (27) avoid attacking active feeding-stage larvae. Apparently the alarm reaction of the prey, coupled with the extrusion of resinous material from their mouthparts, inhibits attack and interferes with feeding when they are attacked. Moreover, such defence mechanisms are more effective when the prey are grouped rather than isolated. Hence, contagion is converted from a liability to a very positive asset by combination with an effective defence.

Other prey characteristics, such as inconspicuous habits and colours, modify the strength of stimulus used by predators in locating prey. The considerable literature on this subject has been effectively reviewed by Cott (14) and clearly demonstrates the survival advantage gained by those prey whose strength of stimulus is lowered by exploiting differences in colour and tone, effects of light and shade, surface continuity and defined contour, and cast shadows and movement. Olfactory as well as visual clues have the same effect, since it has been shown (26, 27) that the strength of odour from sawfly cocoons has a marked effect on the magnitude, but not the form, of the functional responses of small mammals.

When prey are unpalatable to predators, however, the advantage of low strength of stimulus is lost, and it becomes important for such insects to advertise their identity by conspicuous appearance so that predators can

more effectively avoid them. Brower (6, 7, 8), for example, showed that a number of conspicuously coloured butterflies were unpalatable to Florida scrub jays and that the jays learned to associate this unpalatability with the specific colour pattern and thereby avoided the prey by sight alone.

Predators that experienced these unpalatable butterflies also avoided similarily coloured, but definitely palatable, mimics. The avoidance was maintained, however, only in the presence of a preponderance of the unpalatable mimics (8). Hence, when mimicry is involved, predators react not only to the density of mimics but to the proportion of mimics to models in the population. This might well change the form of the functional responses, unlike the other examples discussed, since the instantaneous rate of discovery would be a function of the ratio of the density of mimics to that of models.

Prey characteristics might affect the form of the responses in other ways as well. The behaviour and morphology of individual locusts (1, 56), butterflies (37), and tent caterpillar larvae (62) have been shown to be affected by their own density and by that of their parents as well. Some of these characteristics will undoubtedly affect predation, so that those parameters of the basic functional response equation that are affected by prey characteristics will appear as functions of prey density rather than as constants. Errington (19) feels that a similar situation is the common one when the prey are vertebrates. He argues that predators take a heavy toll of individuals of a vertebrate prey only when the density of prey exceeds a threshold that is determined largely by the number of secure habitable niches in the environment. Below this threshold, the strength of stimulus from prey is so low that there is virtually no functional response to prey density. Above this threshold, individual prey are forced into exposed areas where their strength of stimulus is high enough that a functional response is possible. In such cases, predators remove only surplus prey, ones that would succumb even in the absence of enemies. This effect seems less important when the prey are invertebrates, for their strength of stimulus is often high at all prey densities, and any change in this strength only appears at densities that are greater than those achieved by the majority of insects.

## Conclusion

I have attempted in this review to describe the basic and subsidiary components of predation and to suggest how their effects can be incorporated into mathematical expressions. But is it feasible to express a complete explanation of predation in mathematical language in view of the complexities that can arise? Any such attempt might well be a very rough approximation with limited application and, once proposed, might stifle further understanding in the face of an apparently inviolate mathematical explanation that provides a specific explanation of an event that is actually only generally understood. It is, however, perfectly feasible and practical to explain accurately and specifically the components of simple types of predation and,

hence, to present the explanation of such situations in mathematical form. Since these simple components operate in even complex situations, their mathematical explanation represents part of the explanation of the complex process and, at the least, provides a means to direct effort toward the unknown.

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# BIOLOGICAL CONTROL OF PEST INSECTS IN EUROPE[1]

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Although the first successful utilization of metazoa [by Boisgiraud (86)] and of microorganisms [by Metchnikoff (117)] to control insect pests was in Europe, this method of pest control was further developed in North America, mainly, and in other parts of the world (cp. 28, 181). A recent revival of activity in this field in Europe justifies a separate review covering the work since 1945 in European countries, including western Russia. Since completeness is impossible because of space limitations, the main purpose of this review is to illustrate by recent examples some of the characteristics of applied biological control methods in this densely populated region of the world. Fundamental research and details are reviewed in an extensive article by Franz (61) and will not be considered here. A bibliography, including basic research, of this subject has appeared annually since 1956 in the journal *Entomophaga*.

Only a very few recent European publications are available on details of biological control as a whole [Balachowsky (4); Hinton (83); Szmidt (184)]. Some aspects are treated in books on natural control (104), general ecology (204), and invasions (42). Several reviews on the present status of biological control in the various countries deal also with microbial control, notably those by Franz (55, 59) and Thalenhorst (202) for Germany, by Grison (75) for France, by Kruel (102) and Telenga (196) for western Russia, by Stefanov (176) for Bulgaria, and by Tadić (188) for Yugoslavia.

## Microbial Control

Recent worldwide success in the use of pathogens (179, 192) has stimulated work in insect pathology in Europe. Relatively few laboratories, however, have undertaken practical experiments in microbial control. A thorough review by Krieg (100), which is not limited to Europe, covers viruses, bacteria, and rickettsiae. Other reviews on the practical aspect of insect pathology are concerned mainly with observations and projects in Czechoslovakia (213), France (18, 74, 205), and Russia (49, 50, 129, 130). Literature is scattered over so many journals and printed in so many languages that it becomes increasingly difficult to follow developments.

*Use of viruses.*—A review article by Franz & Krieg (62) deals with viruses of forest insects, including their use in the field; other reviews mention fundamental aspects [Smith (172, 173)], list virus diseases [Smith & Rivers (175)], or discuss possibilities of use in agriculture (174). The first successful ap-

[1] The survey of the literature pertaining to this review was concluded in December, 1959.

plication in Europe of viruses against pest insects in the field was directed against the pine sawfly, *Neodiprion sertifer* (Geoffroy), in Germany [Franz & Niklas (64)]. Although the nuclear polyhedrosis used is a native disease in most European countries, the mortality caused by a spray of an aqueous suspension of polyhedra in the concentration previously used by Bird (14) against disease-free Canadian populations of the sawfly ($10^5$ and $10^6$ polyhedra/ml.) was the same in both regions. This was taken as an indication that even the regular appearance of epizootics was not sufficient in this case to result in the development of resistant hosts or attenuated viruses (56). The introduction of this virus from Scotland to England (Norfolk) was also successful [Smith (174)]. A nuclear polyhedrosis was used with moderate effect against the fall webworm, *Hyphantria cunea* (Drury), in Hungary [Szirmai (183)].

*H. cunea* deserves special attention by students of epizootics, since many more diseases (a nuclear and a cytoplasmic polyhedrosis, a granulosis, three microsporidioses, and some bacterioses and mycoses) appeared in the invaded European countries than were known to attack it in America. The granulosis was used recently in promising field tests in Yugoslavia [Kovačević (95)] and Czechoslovakia [Weiser (217)]. Granulosis was tried without effect against the larch bud moth, *Zeiraphera griseana* (Hübner) [Martignoni & Auer (108)]; it was, however, successful against the European cabbageworm, *Pieris brassicae* (Linnaeus), in France [Biliotti *et al.* (11)] and in England [Smith (174)]. It took several weeks to obtain a mortality of 80 to 100 per cent after peroral infection on third-instar larvae.

Cytoplasmic polyhedrosis was used for the first time against the pine processionary caterpillar, *Thaumetopoea pityocampa* (Denis & Schiffermüller), in southern France. Originally a mixture of nuclear and cytoplasmic polyhedra was sprayed as an aqueous suspension [Biliotti *et al.* (13); Martouret *et al.* (111)]. Cytoplasmic polyhedrosis dominated in the field and was applied by helicopter on 320 ha as a spray (1957) and as a dust (1958, 1959) [Biliotti *et al.* (12); Grison *et al.* (79)]. The production of $254 \times 10^{12}$ polyhedra necessary for such a huge operation was carried out by mass-rearing and by infecting larvae collected from 1300 nests in the forest. Special techniques were developed for rearing the larvae and for preparing the powder [Martouret & Dusaussoy (110)]. The final dosage for application was approximately $12 \times 10^8$ polyhedra/ha. High mortality and heavy infection of the few survivors were observed up to four months after application during the second larval instar. The aftereffects of this treatment to the biocoenosis, particularly to natural enemies of *T. pityocampa*, are under intensive observation. A small scale field experiment in Czechoslovakia against the brown-tail moth, *Euproctis chrysorrhoea* (Linnaeus) [= *Nygmia phaeorrhoea* (Donovan)], also produced high mortality [Veber (210)].

*Use of protozoa.*—The only field experiments using protozoa in microbial control in Europe were carried out by Weiser who has reviewed the subject aptly (214, 218). Of the entomogenous Schizogregarina, Coccidia, Haplo-

sporidia, and Microsporidia, the latter group apparently can serve as a control agent if artificially distributed. It is advantageous that few defense mechanisms occur in the host, and that environmental conditions are relatively unimportant for infections. On the other hand, many of the peroral or, in some cases, transovarial infections are chronic rather than acute, death occurs slowly after infection, and mass production as well as storing of viable spores is difficult. The best methods known to date are to keep the viable spores in water at about 0°C. or to use infected hibernating host insects (for instance, larvae of *E. chrysorrhoea*) as spore reserves [Weiser (215)].

In Czechoslovakia, field experiments using aqueous suspensions of spores *ca.* $3\times10^6$/ml.) of *Thelohania hyphantriae* Weiser against the fall webworm, *H. cunea*, resulted in 25 per cent mortality after two weeks and 100 per cent after four weeks. The effect was not permanent; continuous infection is difficult because infective spores very seldom appear in the host excrement. Predators remove diseased specimens from the crown level where peroral infection occurs [Weiser (215); Weiser & Veber (219, 220)]. A permanent reservoir of several microsporidia pathogenic to *H. cunea* is present in small quantity in larvae of other Lepidoptera living in the same habitat (216). Spraying of orchard trees with spores of *T. hyphantriae* and *Nosema lymantriae* Weiser effectively reduced the number of larvae of the brown-tail moth and the gypsy moth, *Porthetria dispar* (Linnaeus) [Weiser (214)]. *Nosema bombycis* Naegeli, causative agent of the pebrine of the silk worm, is also pathogenic to the fall webworm but cannot be recommended for control operations where silkworms are cultured [Machay (107)].

*Use of bacteria.*—There are very few examples known of successful application of non-sporeforming bacteria against pest insects. Of the several species of *Pseudomonadales* tested in the forests of Czechoslovakia and applied as aerosols from aircraft, *Pseudomonas chlororaphis* (Guignard & Sauvageau) caused a heavy epizootic of the tortricid *Archips crataegana* (Hübner), which spread from the original focus of 0.1 ha to approximately 10 ha [Kudler *et al.* (103)].

Of the sporeformers, *Bacillus thuringiensis* Berliner was described in Germany (8) and first used in microbial control against the corn borer, *Pyrausta nubilalis* (Hübner), in Yugoslavia (116) and Hungary (85). Field experiments continued mainly in France up to the Second World War and were resumed almost simultaneously in Europe against the fall webworm [Klement (89, 90)] and in America against the alfalfa caterpillar, *Colias philodice eurytheme* Boisduval, in 1951 [Steinhaus (178)]. Thereafter, pest control laboratories seemed to realize the advantages of this pathogen and began to test and to produce it extensively. The following tests might be mentioned as effective control was also obtained in the field (European contributions to this are continuing to develop): *P. brassicae* [Krieg (99); Lemoigne *et al.* (106)], also when spores were mixed with fungicides (copper oxychloride or zinc dithiocarbamate) [Martouret (109)]; *Pieris rapae* (Linnaeus) [Herfs & Krieg (82)]; *T. pityocampa* [Grison & Béguin (77); Martouret (109)]; *H. cunea* [Vasiljević

(208)]; *A. crataegana* [Kudler *et al.* (103)]; *Choristoneura murinana* (Hübner) and *Hyponomeuta malinellus* Zeller [Franz *et al.* (63)]; *Arctia caja* Linnaeus, *Heliothis peltigera* (Schiffermüller), and *P. nubilalis* [Martouret (109); and *Malacosoma neustria* Linnaeus [van Damme & van der Laan (30)]. The greater wax moth, *Galleria mellonella* (Linnaeus), proved to be susceptible, whereas careful testing showed that the pathogen was harmless to honey bees [Krieg & Franz (101); Lecomte & Martouret (105)].

Outside of the region under review, field operations against *Dendrolimus sibiricus* (Tshetverikov) using *B. thuringiensis* (= *B. dendrolimus* Talalaev[2]) in Siberia may be mentioned. Here again, as with *P. chlororaphis*, the epizootic spread from small initial foci to extended areas; in addition, spores retained in dead larvae held in the webs served to re-infest the population after hibernation [Talalaev (190, 191)]. This is another way in which pathogens that are artificially produced and distributed might act quite differently from chemical insecticides. Other examples of persistence of pathogens are well-known [Tanada (192, p. 290)]. It is therefore doubtful whether the expression "biotic insecticide" (180) is adequate to describe the actual potentialities of long-living pathogens even if they are only temporarily used.

The important problem of standardization and comparison of different preparations of *B. thuringiensis* has been studied by French workers. The volume of crystals rather than the number of spores indicate the active ingredient per weight unit. Since this is very difficult to measure, a biological unit has been selected as a basis for comparison of $LD_{50}$ of new formulations or strains with a standard preparation; fourth-instar larvae of *P. brassicae* were used as test insects [Burgerjon (23, 24)].

Finally, the application of an undescribed *Bacillus* resulted in approximately 50 per cent additional mortality in white grubs, *Melolontha melolontha* (Linnaeus), in Switzerland. Combining bacteria and fungi did not augment the effect [Wikén *et al.* (223)].

*Use of fungi.*—No other pathogens of insects depend so much on suitable environmental conditions to create an epizootic as do fungi. This is illustrated by the fact that a continuous series of field experiments was carried out in Europe, particularly in Russia, since the end of the last century [for a rather complete list of examples see Baird (3)]; yet very few methods have been adopted in practical agriculture.

Most of the recent field tests were with the genus *Beauveria* (Fungi Imperfecti). Spore suspensions of *Beauveria bassiana* (Balsamo) Vuillemin caused high mortality of hibernating *Aradus cinnamomeus* Panzer (Heteroptera, Aradidae) in Russian forests [Smirnov (171)]. The same fungus was much more effective when applied against the first three larval instars of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), than against last

[2] Several varieties of what is now called *B. thuringiensis* [cp. Heimpel & Angus (80)] and which differ in their biochemical and pathogenic properties have been detected in Europe [Toumanoff & Le Coroller (206)].

instar larvae or adults [Błonska (16); Král & Neubauer (98); Schaerffenberg (157, 158)]. Use of this fungus might become valuable when permanent application of chlorinated hydrocarbons will further increase the tolerance of the beetle to insecticides. Other successful cases include the application of *B. bassiana* against the beet weevil, *Cleonus punctiventris* Germar, in Bulgaria [Stefanov (176)] and against the ermine moth, *Hyponomeuta* sp., in France [Toumanoff & Grison (205)]. *Beauveria tenella* (Delacroix) Siemaszko caused some mortality when applied against white grubs, *M. melolontha* [Wikén *et al.* (223)], particularly, when the soil was humus and not sandy [Schaerffenberg (155)]. Furthermore, experiments using species of *Aspergillus* were effective against the pentatomid *Eurygaster integriceps* Puton [Evlakhova (48)] and against scale insects [Neužilová (119)].

One of the most promising aspects of microbial control is the integration with other (chemical, physical, or cultural) control measures. Most practical experiments in this field were conducted in Russia with Fungi Imperfecti. Chemical control in low dosages, in combination with the use or the presence of pathogens, tends to reduce the tolerance of some pest insects to infection, or to manifestation of diseases [Telenga (196); Telenga *et al.* (199)]. Positive results are reported, for example, from field tests with *B. bassiana* plus DDT against adult Colorado potato beetles and another chrysomelid, *Galerucella luteola* Müller [Dyadechko (39, 41)]; with *B. bassiana* or *Metarrhizium anisopliae* (Metchnikoff) Sorokin, or both, plus BHC against beet weevils, *C. punctiventris* [Telenga (196); Zakharchenko (227)]; with *B. bassiana* plus DDT against the cherry sawfly, *Caliroa limacina* (Retz) [Telenga (196)], the codling moth, *Carpocapsa pomonella* (Linnaeus), in hibernation sites [Dyadechko (40); Telenga (195)], and the caterpillars of *M. neustria*, *H. cunea*, and *E. chrysorrhoea* [Telenga (196)]. Brown-tail moth and beet weevil showed increased mortality and lower egg production, respectively, even in the following generation (198). These and other available observations seem to indicate that several insects are more susceptible to infection after application of (partly) sublethal doses of insecticides and that some sort of synergism may exist between certain fungi and insecticides. The advantage of avoiding permanent high dosage applications of insecticides is obvious [Ripper (139)].

Physical control in combination with pathogens has also been shown to reduce the susceptibility of some insects to diseases. Examples are increased mortality of *C. punctiventris* from fungus diseases (mainly *M. anisopliae*) after treating the field with heavy iron rollers [Nevkryta (120)] or the increase of mortality caused by *M. anisopliae* after application of spores in ditches which trapped the weevil [Pospelov (131)]. Also certain cultural practices, such as humus formation through compost manuring, seem to favor fungal epizootics in white grubs [Schaerffenberg (156)] and other soil insects. Finally, the combination of biological agents (entomophagous insects and fungi) with chemical as well as cultural methods to control the beet weevil have been successful in Russia [Telenga *et al.* (200)].

The conclusion of this interesting field of microbial control is: the artificial initiation of epizootics in insects might be manipulated by the use of better or more pathogens, as well as by the judicious increase of host susceptibility through integration with all other control methods. Therefore, it is suggested that the definition for integrated pest control as given by Stern *et al.* (180) be broadened to include any combination of the effects of beneficial organisms with other types of pest control.

## Use of Entomophagous Insects

The great variety of methods and principles applied in the utilization of entomophagous insects and mites in Europe parallels the diversity of prevailing climatic, agricultural, and psychological conditions. Since fewer pest insects are of foreign origin in Europe than in other continents, the importation of beneficial insects has not been so great. The utilization of native insects, however, is one of the characteristics of European activities in this field. The literature dealing with possibilities and facts of the application of predators and parasites in biological control is very voluminous. This review intends to quote only some typical and recent examples of practical work that might serve as representative guides to demonstrate the present trends. Silvestri (170) gives an excellent picture of the general situation and of rewarding projects for the future. The following review articles on the practical utilization of entomophagous insects in various countries supplement those listed above (p. 183): Czechoslovakian forests [Kalandra (87)]; France up to 1950 [Pussard (137)]; Great Britain [Moreton (118)], Italy [Domenichini (37); Russo (149, 150)]; Poland [Sandner (153, 154)]; Portuguese forests [Azevedo e Silva (1)]; Russia [Rubtsov (142, 145, 146, 147); Telenga (193)]; and Spanish citrus insects [Gómez Clemente (70)]. The books by Rubtsov on biological control with entomophagous insects (141) or on citrus pest insects and their natural enemies (144) treat the subject without limitation to Russia.

*Export of entomophagous insects.*—One phase of biological control is the search for beneficial insects and the shipping of them to other continents. This is accomplished from Europe by the Commonwealth Institute of Biological Control (CIBC), European Station, Delémont, Switzerland, mainly for countries of the British Commonwealth, and by the Entomology Research Division, Insect Identification & Parasite Introduction, European Laboratory, Nanterre, France, for the United States. Through the study of natural enemies of the pest insects that were formerly introduced to foreign countries, these laboratories have contributed substantially to the knowledge of native antagonists of European insects as well as to the technique of handling and shipping them. Practical aspects are mentioned in papers on methods [Delucchi (34); Parker (121)]; on predators of fir tree woolly aphids, *Dreyfusia* spp. [Delucchi (32, 33); Franz (53, 58); Mesnil (115); Pschorn-Walcher and associates (133, 135, 136); on parasites of the fir budworm, *C. murinana* [Zwölfer (229)], the winter moth, *Operophtera brumata* (Linnaeus)

[Pschorn-Walcher & Herting (134)], the spruce tortricid, *Parasyndemis histrionana* (Froelich) [Franz (52)], the diamondback moth, *Plutella maculipennis* (Curtis) [Delucchi *et al.* (31, 36)], the chestnut miner, *Lithocolletis messaniella* Zeller [Delucchi (35)], some Scarabaeidae [Parker (124)], and the lima-bean pod borer, *Etiella zinckenella* (Treitschke) [Parker (122)]; and food plants as well as parasites of some Bruchidae [Parker (123)]. In addition to the work of the organizations mentioned above, experts from abroad were sent to explore and collect in Europe natural enemies of pests that originated in this continent, for instance, of cabbage maggots, *Hylemya* spp. [Wilkes & Wishart (224); Wishart *et al.* (225)], and of fir tree woolly aphids [Wylie (226)].

*Importation and establishment of entomophagous insects.*—In the warmer zones of Europe, characterized by the occurrence of peach orchards or, more specifically, of citrus culture, the introduction of beneficial insects from abroad played a considerable role in biological control. Most results were obtained in subtropical zones along the Mediterranean Sea and in southern Russia, mainly around the Black Sea and Caspian Sea. The following entomophagous insects were introduced (mostly from North America), established, and were of at least some economic value before the time reviewed here (1945): (*a*) In both areas: *Rodolia cardinalis* (Mulsant) against the cottony-cushion scale, *Icerya purchasi* Maskell; *Cryptolaemus montrouzieri* Mulsant against several species of *Pseudococcus; Aphelinus mali* (Haldeman) against the woolly apple aphid, *Eriosoma lanigerum* (Hausmann). Furthermore, several cosmopolitan parasites of citrus pests like *Aphytis chrysomphali* (Mercet) occur in both regions. (*b*) Only in the Mediterranean climatic zone: *Lindorus lophanthae* (Blaisdell) against various scale insects; *Prospaltella berlesei* Howard and *Archenomus orientalis* Silvestri against the white peach scale, *Pseudaulacaspis pentagona* (Targioni-Tozzetti), recently studied in France [Benassy (7)]; *Ooencyrtus kuwanai* (Howard) against the gypsy moth [Templado (201)]; *Aspidiotiphagus lounsbouryi* (Berlese & Paoli) against scale insects [Domenichini (37)]. (*c*) Only in southern Russia: *Sympherobius amicus* Navás, imported from Palestine against the citrus mealybug, *Pseudococcus citri* (Risso).

Of all these above-mentioned entomophagous insects, only *A. mali* occurs also in the temperate climate of Central Europe. There its effect is limited, however, by its late appearance in the spring and its relatively low number of annual generations, caused by the higher temperature requirement for development of *A. mali* than of the host [Borg (19); Evenhuis (45, 46); Šedivý (166)]. The same general experience—the specialized parasite or predator is somewhat more stenokous, i.e., has a lower tolerance for some unfavorable environmental conditions than its host—is exemplified also in *R. cardinalis* on its northern limit in the warmest places of Switzerland (Tessin); there the predator is frequently killed by winter frost, which is tolerated by parts of the host population [Geier & Baggiolini (68)].

After the last war, there was considerable activity in the U.S.S.R. to

import beneficial insects from abroad [Shutova (169)]. This resulted, for instance, in the establishment of *L. lophanthae* against several Diaspididae, of *P. berlesei* against the white peach scale, and of *Pseudaphycus malinus* Gahan against the Comstock mealybug, *Pseudococcus comstocki* (Kuwana), in the subtropical zone of southern Russia [Rubtsov (143, 145); Shchepetil'nikova (168)]. They are reported to be very effective, and further projects are in progress. In Spain, France, and southern Italy, *Leptomastix dactylopii* Howard, a parasite of the citrus mealybug, was introduced from the United States. Its establishment is uncertain but it has been mass produced in Spain, Italy, and France [Gómez Clemente (69); Russo (150); Vayssière (209)].

The San Jose scale, *Aspidiotus perniciosus* Comstock, is a serious economic problem to fruit growers in southern Russia, most Mediterranean countries, and, more recently, in Central Europe. Although *Prospaltella perniciosi* Tower had been introduced formerly into Italy [Russo (150)] and was thought to be a native species in the Caucasus region [Chumakova (26)], this specialized parasite was recently brought to the U.S.S.R. as well as to Central Europe. Russian entomologists used material from the United States as well as the Far East and have reported varying results [Chumakova (27); Rubtsov (142)]. Russian as well as Chinese material was colonized without great success in southern Czechoslovakia [Huba (84)]. *P. perniciosi* from the eastern United States and southern Canada reached France and Switzerland via Germany (Stuttgart), where a mass-breeding station was founded [Schlabritzky (159, 160)]. Although part of the material released belongs to *Prospaltella fasciata* Malenotti, which is native to Italy, different strains of *P. perniciosi*, stemming from the East and the West as mentioned above, are at present being tested in the field in Germany [Klett (91)], Switzerland [Mathys (113)], and France [Benassy (6)]. Survival of some colonies in Germany and France since 1955 makes establishment very probable, but the economic effect cannot be predicted yet.

The fall webworm, *H. cunea*, native to North America, has become a pest of hardwood and fruit trees in southeastern Europe since its discovery in Hungary in 1940. Biological control started with the introduction of this pest to Yugoslavia and the release of about 12,000 specimens, comprising six species of parasitic Hymenoptera and one tachinid, between 1953 and 1955. They had been collected in Canada and the Northern United States by the Commonwealth Institute of Biological Control. A single specimen of one of them, *Meteorus hyphantriae* Riley, was recovered in 1957 [Tadić (187, 189)]. A typically European problem is the necessity of introducing parasites that are compatible with the silkworm, *Bombyx mori* (Linnaeus), because the mulberry tree is also the preferred host plant of the fall webworm. It was shown that the larva of the American tachinid, *Mericia ampelus* (Walker), cannot survive in the silkworm larva [Tadić (186)]. Many native natural enemies, including several diseases (see above), attack the pest in Europe, but the over-all effect is insufficient to stop further expansion. Steps are being initiated to obtain better coincidence of host and parasites by import-

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*Utilization of entomophagous insects: conservation and promotion.*—The most fundamental principle in the utilization of entomophagous insects is to encourage their survival and multiplication. This might be properly called manipulating the environment in favor of beneficial insects. Principles and techniques are the same with native and introduced species. The concept that diversity of cultivated plants reduces the danger of insect outbreaks is widely accepted in European forestry, and the need for "refuge stations" for entomophagous insects in intensively cultivated areas has been clearly stated [Grison & Biliotti (78)]. Fundamental studies of the importance of biotic agents in controlling the level of pest insects in agricultural environments have been stimulated by several unexpected outbreaks and are too numerous to be listed here.

Practical results and experiments aimed at the promotion of particularly important beneficial insects are, however, still relatively rare. Examples include the planting of hedges to give shelter to hibernating *C. montrouzieri* [Gómez Clemente (71)]; the removal of certain leguminous trees near pea fields because parasites of the lima-bean pod borer, *E. zinckenella*, are effective only near the ground, whereas the pest persisted at crown level [Viktorov (211)]; and the protection of the pteromalid *Xenocrepis bothynoderis* Gromakov, an effective egg parasite of the beet weevil, *C. punctiventris*, by plowing without mold-board [Telenga *et al.* (200); Zhigaev (228)].

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It is also part of the conservation and promotion of natural enemies to integrate chemical control with their activity in order to prevent unfavorable consequences of pesticides. Ripper (139) and Schneider (161) treated this subject extensively and showed that through proper timing and alternation of application or choice of dosage of non-selective pesticides, as well as through the use of selective insecticides, the destruction of natural enemies might be avoided. Therefore, only a few additional remarks will be given here.

What is called supervised pest control and practiced by farmers in California and other states recently has been routine work in forest pest supervision in Germany and some other countries for about 25 years [Schwerdtfeger (165)]. No technical control operations are started before the status of natural control factors is carefully weighed. Schwerdtfeger (164) elaborated upon these data and published the first life table for insects in 1932. Proper timing, mostly application of insecticides before the activities of parasites had begun, proved successful, for instance, against the nun moth, *Lymantria monacha* (Linnaeus) [Gäbler (66)], the oak processionary, *Thaumetopoea processionea* (Linnaeus) [Biliotti (9)], the gypsy moth, *Porthetria dispar* (Linnaeus) [Shapiro & Kamenkova (167)], and the spruce sawfly, *Pristiphora abietina* (Christ) [Beier Petersen (126)]. There are many recent examples that indicate that some protection of natural enemies might be obtained by the use of non-selective insecticides without long-lasting effect. This is particularly important where survival of introduced entomophagous insects depends on the judicious choice of or application of the necessary pesticides, or both, as with *A. mali* [Emmel (43); Evenhuis (47); Schneider (161); Sedivý (166)], *C. montrouzieri*, or *L. lophanthae* [Gaprindashvili & Novickaya (67); Sysoev (182)].

Numerous fundamental projects, which cannot be referred to here, were initiated to study the effect of various pesticides on beneficial arthropods. Some new and more selective insecticides and acaricides facilitate integrated pest control. They have proven very satisfactory, for instance, in the protection of acarophagous arthropods in fruit orchards where the red mite, *Metatetranychus ulmi* (Koch), is a permanent menace after pesticide treatment [Collyer & Kirby (29); van de Vrie & de Fluiter (212)]. Clear evidence for the economy of integrated control was given in the Netherlands, where predators of the red mite [*Stethorus punctillum* Weise and *Typhlodromus longipilus* (Nesbitt)] were used in combination with a selective acaricide against parathion-resistant strains of red mites in greenhouses [Bravenboer (20)]. To sum up, the development of integrated control methods is considered to be of particular value to European agriculture and forestry. In such a densely populated continent with a permanent need for maximum agricultural production, this category of biological control has great advantages because it is able to produce reliable results even where climatic conditions are not always favorable to entomophagous insects.

*Utilization of entomophagous insects: mass rearing, accumulation, and*

*colonization.*—The natural distribution of entomophagous insects often does not cover the total area occupied by their hosts. In some cases, mass rearing, artificial accumulation, or field collection and subsequent release of beneficial insects in suitable localities has been able to increase successfully the efficiency of both introduced and native species. *C. montrouzieri*, *L. dactylopii*, *O. kuwanai*, and other foreign natural enemies (cp. p. 189) are regularly mass reared in Mediterranean countries [Gómez Clemente (69); Rupérez Cuéllar (148)], others in Russia. A special technique was developed to winter *A. mali* indoors in England [Massee (112)]. This type of release becomes necessary where conditions are not fully suitable for the permanent colonization of introduced species at the population density required for economic results.

Another method is the transfer within the continent of beneficial organisms to places they have not yet reached by natural means. This "intra-areal introduction" has been applied, seemingly with practical effect, to native predators (Coccinellidae) that became adapted to introduced pests, such as *Chilocorus renipustulatus* Scriba against the San Jose scale, or to new host plants, like *Hyperaspis campestris* Herbst against *Pulvinaria floccifera* Westwood on tea in southern Russia [Bogdanova (17); Rubtsov (145)]. Also the translocation of red forest ants of the group *Formica rufa* Linnaeus from the Alps to the Apennines in Italy is thought to enlarge artificially the distribution area of the predators that already proved effective against the pine processionary, *T. pityocampa* [Pavan (125)]. The same group of ants, mainly *Formica polyctena* Foerster, has been intensively studied in Germany and the Netherlands. Numerous publications have reported on the technique of founding new colonies of this rather polyphagous predator, which has been able to protect groups of trees around natural colonies during outbreaks of lepidopterous pests. A great many recolonizations have been achieved in coniferous and hardwood forests in which a certain minimum number of aphids, which produced the required honeydew, was present. Such nesting hills are legally protected from robbery of pupae by men. The outcome of those experiments, which covered enough ground and were sufficiently long to warrant conclusions, makes it probable that the large-scale colonization of these ants in some forest types is able to protect the trees against destructive outbreaks of some Lepidoptera and Hymenoptera (sawflies). Further experiments are in preparation in order to evaluate more exactly the possibilities and limitations of the method [Escherich (44); Gösswald (72, 73); Wellenstein (221, 222)].

Another way of utilizing entomophagous insects is to accumulate and repeatedly release them in the best possible synchronization with their hosts. All known transitions occur between "inundative release" with a short-time effect and "accretive release" with persistence of influence over several host generations. In addition to many suggestions, some practical experiments that show positive results have been conducted, for instance, with *Dahlbominus fuscipennis* (Zetterstedt) against the pine sawfly, *Diprion*

*pini* (Linnaeus), in Spain [Ceballos & Zarco (25)] and in Poland [Szmidt (185)]; with egg parasites like *Telenomus laeviusculus* (Ratzeburg) against *M. neustria* [Romanova (140)]; *Microphanurus semistriatus* Nees against *E. integriceps* [Ryakhovskij (151)]; and *Telenomus verticillatus* Kieffer against the pine caterpillar, *Dendrolimus pini* (Linnaeus), in Russia [Ryvkin (152)]. In the latter example, parasites have also been concentrated through exposure of host eggs in the field.

Egg parasites of the genus *Trichogramma* have been widely used in Europe. Apart from some experiments to select most active strains [Urquijo Landaluze (207)], trials on integrated control [Baggiolini (2)], and fundamental studies on behavior and physiology of the parasites [Mayer & Quednau (114); Quednau (138)], the majority of recent work has been done in Russia. There is still some confusion concerning the speciation and nomenclature in the genus, particularly since some species have different names in different parts of the world. Hence, no specific names will be given here, with exception of the well-defined *Trichogramma evanescens* Westwood. This species is regularly used on an area totalling 350,000 ha against the noctuids *Agrotis segetum* (Schiffermüller) on beets and *Barathra brassicae* (Linnaeus) on cabbage in the Ukraine [Komosko (92); Telenga (194, 196)]. Similar good results against other agricultural pests have been reported from Poland [Kot (94)]. Further experiments with other species of *Trichogramma* that prefer trees as habitat have been conducted, for instance, against the codling moth, *C. pomonella*, partly in combination with insecticides [Koval'ova (97); Telenga (194, 197)], and against the pine caterpillar, *D. pini* [Ryvkin (152)]. Results seemed to warrant repeated application, although some figures given may indicate that economic effect is a relative value, depending on what growers are accustomed to expect. Mass-rearing methods applied in Russia differ considerably from the techniques common in the United States. In Russia, it is not the great quantity of parasites reared on rather constant optimum conditions that is desired, but a high quality of individuals reared on changing environmental conditions and partly in host eggs more favorable than *Sitotroga cerealella* (Olivier) [Koval'ova (96)]. This selection of harder strains results in a remarkable reduction of the number of parasites required per square unit; this was confirmed by comparative experiments using *Trichogramma* reared on both methods [Stein & Franz (177)].

### Use of Entomophagous Birds

In northern and eastern Europe, mainly in Germany and Russia, birds are not only widely esteemed as effective predators of pest insects, but also artificially colonized in intensively managed forests. In these forests it was possible to increase populations of insectivorous birds five- to tenfold by application of nesting boxes and similar techniques, thereby demonstrating that not food supply but nesting space was the minimum factor [Bruns (21); Pfeifer & Keil (128)]. Several new methods have been developed to find out which insects birds feed on and how much mortality they cause [Franz (54)].

Although it is well-proved that many pest insects are taken, modern experiments to compare the net result of bird predation with other mortality factors are scarce. In some cases, birds did not react density-dependently, i.e., did not take relatively more of a prey when it became more abundant [Tinbergen (203)]. On the other hand, there is some experimental proof that, in forest plots with an artificially augmented population of insectivorous birds, outbreaks of some pest insects either did not appear at all or were of a medium intensity, in comparison with neighboring check plots [Herberg (81a); Schütte (163)]. Observations of population geneticists indicate also that birds might be very effective factors in selecting better adapted moths [Kettlewell (88)]. The present trend, therefore, is to make use of the possibility of increasing bird populations even before the efficiency of this procedure is clearly demonstrated in all cases. There is general agreement that augmentation of such polyphagous predators might help to buffer the impact of an outbreak at its beginning (15, 21, 81, 93, 127, 132).

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# SAMPLING CROP PESTS AND THEIR HOSTS[1]

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## Introduction

Two recent publications discuss the philosophy and technology of insect population sampling. Morris (128) did not attempt to present a complete bibliography on sampling and sample extraction mechanics, nor did he say very much about agricultural crops and the pests that feed on them. Balogh and his collaborators (11), on the other hand, dealt extensively with sampling methods appropriate to all forms of land animals, from soil protozoa to mountain lions.

The present article is complementary to Morris' in that sampling and extraction mechanics are reviewed in some detail and recent work on plant hosts is considered. It is complementary to Balogh's in that an attempt has been made to indicate the proportion of the available population actually sampled by a given method and to compare the results obtained when contrasted methods are used to assess the same species. A few statistical principles, mentioned by Morris but apparently not dealt with by Balogh, have been re-emphasised and interpretive difficulties discussed.

A deliberately extended list of references covers many of the problems dealt with by agricultural entomologists; this was completed in March, 1960, and, except for about a dozen items where specific points have been emphasised, it excludes papers and textbooks cited by Morris. By the same token, 14 of them appear in Balogh's comprehensive bibliography.

## Reasons for Sampling Crop Pests

Applied entomologists are ultimately concerned with preventing pest damage; increasingly large areas are now sprayed or dusted every year, and the ecological consequences have stimulated an increasing interest in rational chemical and biological techniques to maintain the present high level of crop production and minimise undesirable biological side effects. Within this field many problems arise which can only be solved by analysing sample estimates of pest and host abundance and activity.

Pests are usually sampled so that their abundance can be predicted, the losses attributable to them measured, and their damage prevented. These aims require different approaches: attempts at prediction or at direct control by biological means demand detailed work on the survival and emergence of overwintering forms, their dispersal to and activity within host crops, and the natural enemies and other mortality factors that affect them throughout

[1] The survey of the literature pertaining to this review was concluded in March, 1960.

the year. A common reference point is useful where so many different measurements have to be integrated, and results are most conveniently expressed in absolute unit area or volume terms (85).

Loss appraisal on a regional or national basis is often a matter of collective opinion, or of rank sampling, based on extensive survey visits to a suitable number of host crops (7, 75); but it is less easy to estimate the numbers of insects a crop can tolerate without serious loss of yield, and an intensive field experimental approach is needed in studies of relationships between pest numbers and yields. Such results, combined with extensive survey data, can give reasonably good national estimates of pest status and damage (162).

Direct prevention of crop damage is usually studied on field plots; thus, natural enemies may be released in some areas (which may be considered very large plots) but not in others, while the potential use of a new varietal cross or chemical spray can be demonstrated by the difference in pest population, symptom expression, or yield between quite small treated and untreated plots. There is a basic distinction between loss appraisal and direct control work, where differential estimates often suffice, and more detailed ecological work, where absolute estimates may be needed.

The specific aims of an investigation determine the design of the sampling programme; but there is little point in the entomologist appealing for help with design unless he can tell his statistical colleague precisely what is to be measured. He must, for instance, decide what sort of sample is likely to provide the information he wants, whether indices of population density obtained by rapid examination at many localities will be more informative than slow but accurate counting of individual specimens at one or two sites, and whether some form of pest or host activity measurement is needed. He must also give the statistician some guidance on the level of accuracy required: it is much easier to be right 95 times out of 100 about a doubling or halving in population density or damage than to be right 99 times out of 100 about a 25 per cent shift.

There are many applied problems where estimates of successive differences in growth or population density are more interesting than estimates that refer only to a given place and time. Such successive estimates are often difficult to analyse and interpret because many biological processes are influenced by seasonal and local changes in weather, and the separation of the activity component from data that are effectively an integration of population density, activity and growth, weather, and time is a complex matter (23, 66, 88). Deciding how to treat the temporal component in analysing activity rhythms presents special difficulties (37). Because of these complications, it is worth considering relatively simple static sampling problems before discussing the measurement of growth and other forms of activity.

## Sampling in Space

There is no need to recapitulate Morris' summary of sampling methods here, but it is worth commenting on a few points which, in my experience,

many entomologists find hard to grasp. Sampling efficiency, for example, is a product of statistical, economic, mechanical, and personal components. The size and number of individual sample units, their destructive or non-destructive nature, and their efficient allocation over the study area contribute to statistical efficiency in the sense that the best estimate has the smallest variance; but, as Yates (196) points out, the optimal sampling method is the one in which the total cost of the work is minimised in relation to the accuracy required.

Decisions about the size of individual sample units are not always easy because the most efficient size depends on the way in which the sampled organisms are spatially distributed, and it is difficult to detect the underlying distribution from a preliminary series of small samples: some kinds of non-random arrangement appear random if the sample units used are too small or too large (1, 73), and it is sometimes worth sampling quite a large contiguous area in a grid pattern (18, 173) if detailed information on distribution is needed.

Except at rather low population densities per unit area, and sometimes at high densities, insects tend to be found in aggregations; even within species, a sampling procedure optimal for one instar may not be suitable for later stages in which dispersal leads to a more uniform distribution (29, 81, 91, 182). This is because characteristics of insect population distributions are not necessarily intrinsic properties of the species but may be altered by microhabitat variations (44, 142, 148) or imposed by the growth form of the host (148).

Where the distribution of specimens is of interest, the data may be fitted to one of two kinds of mathematical model. The Poisson distribution is a good example of a theoretical model to which no innate biological meaning is attached; Finney & Varley (59) generated a Poisson series even though they knew that the eggs in which they were interested were not randomly distributed, and they demonstrated that egg mortality was random with greater precision than was possible with a more complex model in which some of the sample information would have been lost in estimating more parameters. Others (56, 167, 185) have used Poisson models to assess the adequacy of various sampling methods.

The second kind of model contains constants to which some biological meaning can be attached. These are useful in detecting over-dispersed populations but are often difficult to interpret (18), even when sampling has been quite intensive. Thus Anscombe (8) was unable to estimate the exponential $k$ (which is thought to depend on the intrinsic power of the species to reproduce) very accurately from 940 sample units of black aphid eggs, and he suggested that the real distribution of the eggs may depart from the model which gave the best fit to the observed data. More complex models, such as those designed to test theories of population growth or activity, are sometimes of limited use because more than one plausible biological meaning can be given to a constant [Skellam in (131)] or because, with present knowledge, the coefficients cannot be given precise meaning (121). It has recently been

suggested that models that attempt to describe specific phenomena have less merit as research tools than meta-models that define the kinds of reactions that take place in general biological systems such as food-chains or community structure (114, 151).

When the aims of a study have been defined and unit size of sample agreed upon, a decision must be made on the most efficient sampling process. Thus, Le Roux & Reimer (106) studied the optimum numbers of samples needed for efficient population estimates when predator, parasite, and prey have to be sampled concurrently; of course, optimal allocation of units over the study area is essential for statistically comparable estimates at minimal cost. The same principles apply in extensive and intensive work: Davis & Wadley (43) showed that worth-while reductions in the annual cost of a grasshopper egg survey could be achieved by stratifying sample units within groups of counties, while Ives (90) allocated branch samples in sawfly work on the basis of crown depth and form.

## Sampling Host Plants

There are excellent books on surveying and measuring vegetation (24, 73), and there is no need to describe the methods here; a note on recent work will suffice. New forms of multifactorial plant association analysis are being developed which emphasize relationships between species (191), methods which may be useful in soil fauna work and other studies where animal communities and their food-chains are concerned. These relationships are at their simplest in pure stands, such as weed-free crops, and become progressively more complex through cultivated and wild pastures to natural woodland. From a sampling viewpoint, the variability found within and between crops is important because the errors in pest and host sampling are additive. Leads to the literature include papers on grassland (95, 124, 179), cereals (10, 196), potatoes (52), beans (152), broccoli (126), onions (140), fruit (34, 92), and the partitioning of variance components in work on horticultural crops (76). With tree crops, Lindsey and his colleagues (110) have estimated the relative efficiencies of nine forest sampling methods in terms of field time required to obtain estimates with 15 per cent standard error, and others (92, 149) have investigated optimal sample size for estimating fruit quantity and quality in apples and pears.

A tradition exists that plant tissue subsamples are comparable among themselves, but sometimes this assumption is not justified (20); subsample size should always be checked, and the botanical literature is helpful in suggesting methods for estimating leaf areas (2, 61, 123), classifying leaf shapes (28), and measuring areas of fruit skin (25).

Yield estimation is important in work on the pest infestation levels at which control is uneconomic. Counts of damaged and undamaged fruit are straightforward (35), but yield samples in field crops often fall between the volumes of vegetation that can be handled by farm machinery and those that can be dealt with on the laboratory bench. Equipment has been de-

veloped to handle trial-size samples without appreciable losses; forage crop harvesters (82, 98), threshing machines (13, 193), a seed cleaner (159), and electronic devices for counting and sorting seeds (70, 174) are available. At the other end of the scale, there is a precision seeder for field studies on spatial relationships (166).

## Sampling Insects

Insects, for purposes of sampling, can often be regarded as fixed points in space, and some of the methods devised originally for botanical work have been used in entomology (44, 60, 131, 150). Whatever method is used, the chore of separating the insects from their hosts or habitats and counting them remains; the rest of this section deals with separation and counting methods that can be applied independently of insect activity at the time the samples are taken.

*Direct assessment on host plants.*—Direct assessment should be a goal, because neither the insects nor their hosts are destroyed, a point which is often overlooked until the worker begins to wonder what effect a destructive sampling method has had on his estimates, which refer to an ecosystem that he had progressively altered.

Ranking by symptom expression or severity of infestation gives rapid assessments: aircraft have been used to estimate the extent of insect damage and of breeding sites over large tracts of country (5, 83). More commonly, field estimates are made of the proportions of plants infested at varying levels of severity, and the results are expressed either as frequencies or in integrated terms after transformation (7, 72); but subjective elements can bias results (33), and systems usable in one season may be of little use in other years when pest or damage incidence is different (72, 161). Further, ordinal class numbers do not give reliable figures for analysis, though this difficulty can to some extent be overcome by subsampling each rank and weighting the frequency data proportionally (12, 161, 181). There are also ways of transforming variables that cannot be measured on a precise scale into figures amenable to statistical analysis (22).

Reasonably precise estimates of population density or damage can often be obtained by direct counting, though care must be taken to relate symptom expression to pest density when the one is easier to count than the other. Graham (71), for example, has shown that the relationship between numbers of injured fruit, numbers of injuries, and numbers of codling moth larvae varies with locality and season; there is a similarly loose relationship between frit fly larval population and numbers of visibly damaged oat shoots (163).

Counting is tedious, and personal errors may bias results. Buckner (27), who argues that total reliance on one method is unwise, has reported that lack of agreement between two methods could be caused by the collector searching more diligently at low than at high levels of population. Any method that reduces field counting, particularly in adverse weather, is use-

ful, and the same holds for plant samples brought back to the laboratory for examination. Curtis (41) avoided removing embedded eggs by using a differential staining method, and similar techniques have been developed for plant nematodes [(69); Minderman in (131)]. Aural (4), optical (50), and flotation (103, 188) methods are used to detect pests or their damage in seeds.

*Separation of insects from plant tissues.*—Samples may change in faunal composition if there is appreciable delay before examination, and mechanical extraction from host tissues gives biased results if a constant proportion of the fauna is not collected and if specimens are damaged beyond hope of identification. Probably the least harmful method is beating, which gives good comparative population estimates if care is taken; thus Summers & Baker (164) corrected mite counts for inequalities in twig subsamples, while Wada (180) derived a formula to give total population estimates from counts of mites dislodged by beating cedar twigs three times on a collecting board.

Beating is not as reliable with active insects; at air temperatures exceeding about 60°F. some beetles fly away from jarred branches and escape capture (153, 194), and it is difficult to beat a random sample of branches on a tall tree. Some workers (38, 153) spread sheets under tented or untented trees prior to pyrethrum treatment, and the knock-down effect allows much of the fauna to be collected. R. C. Muir (*in litt.*) has recently tested the efficiency of the knock-down tent method: he put known numbers of marked mirids on the branches of a tented apple tree a few minutes before treatment; 60 per cent of the marked specimens were recovered from the ground sheet, the remainder being lodged in cracks in the bark, particularly at the union of branches and main stem.

Good results have been obtained by brushing insects from host tissues (31, 102, 118, 127). This method is probably more effective than leaf imprinting (31), particularly when the specimens are dispersed over different sorts of host tissue (164). Both brushing and imprinting, however, have been criticised in aphid work because of the damage done to soft-bodied insects (112).

Beating and brushing depend for success on the extent to which insects release their grip on host tissues in response to mechanical stimuli. Physiological stimuli are also used in sample separation; thus, light and temperature reactions are used in Berlese-type funnels, but care must be taken to avoid over-heating with consequent desiccation and death of specimens before they reach the collection jar (171). Olfactory and gustatory reactions are used: methyl *iso*-butyl ketone works well with some aphids (81), and turpentine extraction of thrips is satisfactory for some stages of certain species (109) but less useful for others (171).

More vigorous separation processes employ centrifugal force, aspiration, and washing. Harvey (77) extracted 10 per cent of the mites from a mass of curled wheat foliage and webbing by centrifugal treatment, and Minderman (in 131) has adapted a centrifugal flotation method for nematode work.

Various essentially similar types of suction collector are used for collecting insects from ground vegetation and tree foliage; Johnson and his colleagues (94) obtained good extractions of Hemiptera, adult beetles, and springtails with a modified electric vacuum cleaner, but their machine only collected 70 to 80 per cent of the millipedes, centipedes, and fly and beetle larvae. This sort of collector has been modified and made independent of electricity (48), and a truck-mounted version has been used to collect very large numbers of parasites for release elsewhere (176). Kennard & Spencer (99) use a pole-mounted vacuum collector for sampling insects on tree foliage and flowers, but they consider their results to be quantitative only on a comparative basis.

Direct washing is sometimes used to separate aphids from distorted leaves (33, 104), and the addition of chemicals to the water can improve efficiency; thus Newell (132) added monoethanolamine oleate to extract mites from orchard foliage, and others have used hot KOH for mites (96), water-detergent mixtures for thrips (171), and petrol for eggs (39).

Various indices have been used to estimate insect population density where direct counting is difficult or impossible, and these have been reviewed by Morris (128) and Paramonov (137).

*Separation of insects from soil.*—Two recent compilations on soil zoology (101, 131) deal with many aspects of sampling and sample extraction, and the following comments emphasise a few points relevant to work on soil pests.

First, unless the species is restricted to a known habitat or patches of obvious crop damage can be identified before sampling, within-field sample stratification is not possible. Sampling must thus be random or systematic, and there is much to be said for a combination: the field is divided into roughly equal parts, and sample units are taken at random in each part [(32); Healy in (131)].

Secondly, the right sort of sampling tool depends on the insects under study: core augers are commonly used, though nematode samples are often taken with corkscrew or half-cylinder augers (56). Large numbers of small samples have advantages in greater statistical efficiency and in reduced volumes of soil for examination; in routine work such soil samples can often be bulked, and coned and quartered after thorough mixing if the bulk is too great for easy extraction. Sample size has tended to decrease: Burrage & Gyrisco (30) found that 1 sq. ft. units were twice as efficient as 4 sq. ft. and three times as efficient as 9 sq. ft. units in chafer work; a 4 in. core has been used in Britain since 1939 in preference to 6 sq. in. wireworm samples (57). Hughes (89) has used a 2 in. auger in an interesting new method for estimating anthomyid pupal densities, while Macfadyen (115) recommends very large numbers of $\frac{1}{2}$ in. cores for sampling soil mites.

It is difficult to be certain that all or even a constant proportion of the animals have been separated from their soil or litter substrate. Sometimes hand-searching is adequate but can give misleading results even with in-

sects as large as elaterid (36) and scarabaeid (29) larvae; the alternatives are flotation, wet sieving, dry funnel extraction, or the use of physical or chemical irritants. Funnels extract slowly, introduce errors due to changes in sample composition with time, only extract living and motile forms, and are differentially efficient with soil type and species. Wet methods are rapid, extract living and dead forms, which are not possible to distinguish if a lethal boiling or benzene final separation process is used, and are generally inefficient when much organic matter is present in the samples. Other methods are useful when comparative estimates of quite large species are wanted: thus, although it is not yet possible to define the limits of soil volume over which electrical methods are effective, these can be used to assess differences in earthworm population density [Nelson & Satchell in (131)]; permanganate solutions will also cause some species of earthworms to emerge from their burrows (165); orthodichlorbenzene emulsion causes about 85 per cent of the tipulid larvae to emerge from their burrows under certain conditions (122); and a kerosene-pyrethrum mixture similarly extracts about 13 per cent of the salt marsh tabanid larvae (183). Pitfall traps are used to sample forms that are active on the soil surface (11, 190).

*Counting large numbers of insects.*—Squared and sectored plates simplify counting insects after separation from host tissues or soil (69, 127, 160); an aliquot part can be counted where very large numbers are present. Investigating this possibility, Morgan and his colleagues (127) found that mites were not uniformly dispersed over sector grids, so they adopted a sequential counting scheme based on a 10 per cent error in estimation. Strickland (160) found small observer differences in sector counts of aphids; populations of 1000 or more could be estimated with a 10 to 12 per cent error by counting one-twelfth of those present. More uniform dispersal of specimens would, of course, reduce these errors; polyvinyl alcohol (102) and the addition of glycerol to alcoholic suspensions [Murphy in (131)] help dispersal and tend to prevent accidental movement of specimens during counting.

Volumetric and gravimetric methods can simplify counting difficulties (12, 107, 133), and a turbidimetric method estimates the numbers of nematode larvae in a suspension (17). Gross volume of specimens can be misleading because of compaction at the bottom of the measuring cylinder, and with Hemimetabola there are differences in weight and volume between nymphs and adults (160).

Sorting and counting have also been done electronically. Holling (84) used radiography to distinguish healthy from parasitised sawfly prepupae, and H. E. van Emden (*in litt.*) is developing a radiographic method to detect dipterous stem borers within cereal and grass shoots. Lowe & Dromgoole (112) have made a machine which will count aphids in water suspension with a repeatable accuracy of $\pm 3$ per cent.

## Sampling in Time

The sampling problems and methods considered so far assume that the sampled attributes are fixed points; but, except for periods of dormancy,

living things grow, and most insects move about as well. Plant and insect growth and insect dispersal problems are both temporal functions and can properly be considered together.

*The measurement of plant growth.*—Plant growth is an integration of many environmental components, and much of the work on its measurement has been done in the laboratory where these can be studied separately. From an applied viewpoint, the effect of growth rate on the extent of the photosynthetic surface is of interest since variations are thought to affect productivity more significantly than the rate and mechanism of the photosynthetic process itself (123). Yield is also relevant, and a preliminary working hypothesis has recently been published (156).

Watson (in 123) has suggested that big increases in leaf area late in the season, when dry matter assimilation is declining, may give greater over-all dry matter yields but, in many crops, no increase in economic product. Growth form influences effective leaf area: the lower leaves of an open crop, like kale, receive sunlight below the intensity at which their conversion potential is fully used; beet, on the other hand, attains its maximal growth rate at a much greater effective leaf area than kale, possibly because the leaves are bunched and less shading occurs. Host growth pattern influences the numbers and distribution of some pests (148, 168) and is important in studies on relationships between pest numbers and yield. Field estimation of leaf shapes and areas (2, 28, 61, 123) is important here, as are successive measurements on marked plants; the latter are often difficult with annual crops in the field as continued handling causes damage and repeated visits to the same plants in wet weather leads to soil compaction, puddling, and other complications that may vitiate the results.

While variations in leaf area are important in productivity studies, entomologists sometimes measure plant growth for other reasons. Morris and his co-workers (129), for example, use shoot elongation as a phenological index; other workers (16, 105) use phased development charts for rapid growth assessment in the field; and Luckman & Decker (113) devised a tassel index to measure corn plant maturity in European corn borer work. Tree growth is estimated by trunk or branch girth measurements or with trunk augers (120); there is a relationship between girth and gross weight (138), crown projection, yield, and age (178) in apple trees.

*The measurement of insect activity.*—Movement in active insects is often measured by appropriate devices in the laboratory (26, 53, 184, 187) because external influences can be controlled. Field measurements, in contrast, are confused because population density, behavior, weather, and mechanical efficiency influence the results.

*Light traps.*—Trends in light-trap design have been reviewed by Frost (63). More recent work has been done on the attractant efficiency of light at different wavelengths, and Pfrimmer (139) concludes that each group of species is a separate problem requiring careful study to find the best type of trap. Much has been written about sex- and species-differential catches. Argon lamps collect more pink bollworm moths and a higher proportion of

females than black light (68); whereas fluorescent black light is very attractive to chafer beetles, and visual observation suggests that such traps catch about one-third of the adults active within trap range on a given night (170). Early attempts to use light traps to control moths seem to have been abandoned because, although the catches may reflect the relative numbers of a species, they cannot prevent 100 per cent infestation levels (119, 135, 154). Highly significant reductions in mole-cricket populations have been reported in light-trap trials in Egypt (87).

In a study on the interpretation of light-trap data, Provost (141) compared catches of mosquitoes and moths in unlit, and apparently objective, air-sampling devices with catches in standard New Jersey traps. He concludes that light-trap catches are the result of a purely physical cycle in which attractant efficiency is superimposed on activity patterns that are regulated by minute changes in natural night illumination. Loomis (111) investigated the trapping efficiency of air-flow mosquito light traps: after running for several weeks in the field, the traps operated at less than half their maximum air-flow capacity because accumulated insect debris impeded the screen funnel and motor mounts; in test rooms, where known numbers of mosquitoes were released, 55 per cent were caught in traps with an air-flow of 238 ft. per min., and 26 per cent with an air-flow of 113 ft. per min. It is clear that light traps do not catch a particularly high proportion of the insects that come within attractant range, and it is interesting to speculate about possible behavioural differences between the trapped and untrapped fractions of the population. An important contribution on mosquito traps has appeared just recently. Haufe & Burgess (77a) found no significant differences in the ratio of day to night catches between patterned visual and objective suction traps. The visual traps caught about 10 times as many mosquitoes as the suction traps, and the authors suggest that their black-and-white pattern was superior in contrast to competing patterns in the environment.

Colour enters significantly into trap design, and Moericke (125) has concluded that white surfaces are avoided by many insects in search of food plants; but adult frit flies respond best to white plates (A. Ibbotson, *in litt.*), and white water traps strongly attract several species of thrips, which feed mainly on flowering plants (108). Many aphids are strongly attracted to yellow traps (100), which also seem to catch most Japanese bettle adults (189), whereas European chafer adults prefer black and red (169). With flies the position is also confusing: mosquitoes respond best to yellow and orange (65), simuliids to dark blue (42), and tsetse flies to "natural," as distinct from black, hessian (175). Sound may also play a part in trap efficiency and has recently been used in mosquito work (192).

*Bait traps.*—Insect attractants and repellents have been reviewed by Dethier (45). Two types of attractant are used in insect survey and control work: sexual scents, particularly with certain moths (3, 45), and food odours. Food baits vary from raw plant materials (40, 86), through hydrolysed

plant proteins (136), to refined chemical attractants like ammonium carbonate, metaldehyde, and methyl eugenol (15, 45, 177). There is now evidence that host specificity is mediated by secondary plant substances that are highly attractive to different pests; thus a glycoside extracted from several different plants is highly attractive to tobacco hornworm moths and Colorado potato beetles (195).

Bait attractiveness is often as hard to assess as catch data are to interpret; position, sex, and species-differential effects, apart from bait composition, must be considered, and care is needed even in interpreting comparative results (51). Aromatic baits do not trap all of the specimens they attract (9), and some fly traps increase in attractiveness after the first few specimens have been caught (14, 46).

It is interesting to compare catch data obtained by bait and other forms of sampling. Groves (74) was unable to reach firm conclusions about the relative merits of bait and light traps in codling moth work, while Alexander & Carlson (6) found that baits gave lower codling counts than rotary nets early in the season. Steiner (157) quotes recapture of 62 oriental fruit flies out of 405 released in a test of methyl eugenol baits, but 30 or more bait stations per square mile gave 60 to 100 per cent control of male fruit flies in Hawaii (158). Van den Bruel & Moens (177) compared superficial searching, dry sieving of soil from random quadrats, collection of dead slugs after dinitrocresol (DNOC) treatment, and collection of paralysed slugs after metaldehyde baiting. Quadrat sieving gave the best estimates of population density for the commoner species, while the two chemical irritants gave contrasted species-differential surface activity estimates. MacLeod & Donnelly (117) devised a tent trapping method to give unbiased estimates of blow-fly population; these estimates directed the authors' attention to sex and position biases in their bait trap data.

*Impaction traps.*—Coloured or plain impaction traps are widely used in field studies because of their cheapness and simplicity, but again interpretive difficulties arise. A water trap, for instance, catches more aphids than a flat sticky trap of equal area, which in turn catches only about half as many as a cylindrical trap of the same area (80). Sampling power is partly a matter of wind speed, and at low wind speeds alighting behaviour is important even in the absence of attractant paint (C. G. Johnson, *in litt.*). Catch thus represents a product of wind speed, behaviour, and insect density per unit volume of air, and must at least be divided by wind speed across the trap if population density estimates are needed; where direct anemometer readings cannot be made, theoretical wind profiles can often be used to correct raw data before analysis (172).

Trap size affects catch and, although catch per unit area of trap surface is inversely proportional to size, it is usually best to use as large a trap as possible because greater numbers of specimens provide better figures for analysis (80, 155). The trapping medium is also important: water traps catch more aphids than sticky plates because the sticker tends to obscure

the attractant paint; but water traps have to be visited every two or three days in summer, and their use in survey work is thus restricted (103). Stickers vary in retaining, optical, and weathering properties (64, 79, 155), and, in recent work on frit fly, I found that catch was proportional to Stictite® grease dosage up to 0.4 gm. per sq. in. of trap surface. Heavy grease dosages are also recommended in boll weevil work (64). Where trap colour is important, catch can reduce efficiency: 12 to 14 frit flies per sq. in. of trap surface reduce the efficiency of white plates by about 80 per cent (A. Ibbotson, *in litt.*).

*Suction traps.*—Suction traps sample a constant volume of air per unit time, are to some extent independent of wind speed, and are probably non-selective (93), at least with weak-flying or drifting insects, which cannot take avoiding action once within trap range. Several comparative studies have been made: Johnson (93) concluded that suction trap aphid-density estimates are likely to be more accurate than sticky or tow-net trap estimates; Heathcote (79) obtained suction trap aphid catches with a smaller variance than he found with water or both cylindrical and flat sticky traps; Lewis (108) similarly got more consistent thrips catches with a suction trap than with sticky or water traps when all were sited at crop level.

*Sweep nets.*—Light and suction traps are often operated, with or without time-clock sorting attachments, for quite long periods and require reliable power supplies; bait and impaction traps also operate continuously without attention. During such trapping cycles insect activity may change because of weather (88), and population density may change because of variations in natural mortality. Sweep nets, because of their simplicity, are often used to obtain rapid estimates of pest distribution, activity, and abundance (11, 54, 185), especially where power is not available to run, and topographical features limit the range of, more sophisticated apparatus. Flushing is also sometimes used for large, easily visible insects.

Sweeping is easier in some crops (such as Gramineae) than others (leguminous crops, for example, may be damaged in the process). It is ineffective when vegetation is wet and at wind speeds exceeding 4 to 5 m.p.h. There are also problems of subjective bias caused by individual variations in height, length of pace, and strength of sweep, while, with some biting flies, net catch may reflect the reactions of the insects to the behaviour of the operator (60). Some of these criticisms may be less important when mobile nets of the sort developed by Read (143) or Nicholls (134) are used.

Several studies on the interpretation of sweep-net catches have been made. Hughes (88) investigated the influence of prevailing weather on catch, and Richards & Waloff (146) noted that young grasshopper nymphs were less efficiently caught than later instars: over a series of tests, net catch varied from 20 to about 40 per cent of the available population as estimated by an "absolute" method. Dietrick and his colleagues (47) compared sweep-net catches with data obtained by suction collection. They adjusted their results to a standard unit area basis and showed that their apparatus col-

lected between $1\frac{1}{2}$ (adult spotted alfalfa aphids)to over 300 (borborid flies) times as many specimens per unit area as their sweep nets. R. L. Willis (*in litt.*) estimates that about 40 per cent of the potato psyllids can be swept from infested plants. Land-Rover flushing efficiency has been estimated as 60 per cent with the red locust (147).

*Emergence cages.*—Cages are used to estimate spring emergence and to study activity in confined populations; thus Dobson and his co-workers (49) used a large terylene cage to enclose a wheat plot. Frick (62) found that small cages gave cherry fruit fly emergence figures a day or two later than bait traps; similar results were obtained with codling moth in Holland (55), where the cause was thought to be the absence of direct solar radiation because of screening by the traps themselves. Conical zinc cages, in contrast, gave results that correlated well with field observations on gall midge emergence (133).

*Marking and recapture.*—Population density, growth, and activity have been estimated by recapture of a proportion of specimens previously marked and released. Morris (128) gives some leads to the literature, and a few general comments will suffice here. It is, for instance, difficult to fulfil the basic assumptions of recapture theory in practice: there is an inevitable time lag between capturing, marking, releasing, and recapturing, and some newly emerged insects are more sensitive to handling, anaesthetisation, and marking than older specimens. Little information is available on changes in behaviour with age, which might bias the chance of subsequent recapture, on the ethological effects of tagging with doses of radioactive tracers strong enough to allow individual insects to be subsequently identified, or on the precise areas over which recapture is effective and, consequently, on the size of the studied population.

Some attempts have been made to assess the errors in recapture data. Thus, Richards & Waloff (146) compared their pooled grasshopper population-density results with data from "absolute" quadrat counts; the estimates differed by an average of 28 per cent, the range extending from a recapture estimate 66 per cent higher than its paired quadrat counts to a quadrat estimate 46 per cent higher than the recapture figure. Muir (130) attached a 27 per cent standard error to his mirid population estimate, and MacLeod (116), working with low incidence blow-fly recaptures, estimated that 37 per cent of the marked flies remained within trap range for a week, and that 25 to 50 per cent of these were in fact recovered.

## Discussion

Applied entomologists spend much of their time in the field studying pests, their hosts, and associated insects, and sampling problems and the analysis and interpretation of sample data are nowadays factors to be reckoned with in allocating time and resources. It is therefore worth concluding this review with a short discussion of some of the procedural and interpretive difficulties that arise.

*Field experiments.*—Many considerations—area of land available, host plant and soil variability, the need for realistic yield data, ease of access, and so on—influence decisions about plot size and treatment replication, and recent work by Hatheway & Williams (78) on the efficient estimation of plot size in relation to costs and yield variability provides a useful starting point when complex field trials with insects are mooted. But all too often plot decisions are made without considering pest dispersal and behaviour; this is unrealistic, particularly where likely benefits from spray treatments are to be measured. Joyce & Roberts (97), for example, demonstrate the dangers in assuming that the insect complex on a 10 feddan (1 feddan = 1.038 acres) cotton field within a 90 feddan area of cotton is not affected by other treatments applied within that area. Independence can probably be assumed in 20 feddan fields separated by at least 150 metres. Broadbent (21) has argued against wide, anti-drift, guard strips because greater crop variability can be expected between widely separated plots, but there are cases where relatively low insect population densities, coupled with marked habitat variation, necessitates work on plots as large as $\frac{1}{2}$ to 1 sq. mile (145, 167). Compromises between host and pest variability often have to be made, and rational rather than arbitrary decisions will simplify interpretation of the results.

*Trapping estimates.*—In many agricultural problems the fact of pest dispersal or presence is significant, and this can often be shown quite simply by trapping or sweeping; but even where the required information relates to the first discovery, rather than to the mean of the observations, it is clear that the wrong sort of trap or emergence cage can give misleading results.

In long-term activity problems it may be useful to interpret results in the light of absolute population density estimates, and Webster & De-Coursey (186) have derived a mathematical model to estimate total population from trapping data; this model assumes that the conversion of an insect population from a "wild" to a "caught" state follows the principles of a chemical reaction converting one substance to another, and also that the rate at which insects are caught is proportional to those remaining untrapped. Skellam (in 131) has considered the implications of different assumptions made in interpreting incomplete trapping and extraction data; inefficient trapping and heterogeneity of response in space and time greatly influence the sort of mathematical model appropriate to estimating absolute population density from incomplete records. Skellam illustrates these difficulties with three biologically plausible graduations based on published records from which the original authors inferred a total population of 485 individuals. The graduations gave estimates of 540, 700, and 1000 individuals respectively, and the last two provided better fits to the raw data than did the original and the first. Unless the validity of the underlying assumptions can be confirmed, it is difficult to see how far trapping records can be interpreted other than differentially.

*Growth assessment.*—Growth tends to be exponential and cyclical, and quantitative estimates are often transformed to a suitable scale so that the resulting straight or curved lines can be tested for goodness of fit against a derived mathematical model; cyclical data are often smoothed by striking running averages before analysis. The whole subject is fraught with difficulties and pitfalls for the unwary, especially where age trends in small populations are concerned; Cole's (37) work on cyclical rhythms in the unicorn, Bliss' (19) study of periodic regression in biology, and Glenday's (67) work on growth curve analysis are relevant here.

*Analysis and interpretation.*—The statistical, mechanical, and personal errors associated with much practical insect and crop sampling seem to be of an order ($\pm 20$ to 30 per cent of the mean values) sufficient to detect a doubling or halving of the population being studied, and, because many of the field processes that interest applied entomologists are multiplicative, a factor of 2 is often sufficient to enable major trends to be identified. Subjective judgment over curve-fitting to graphed results (58), and simple statistics such as mean values with their associated standard errors, are frequently all that is needed to describe sample data, but even at this level interpretive difficulties arise. Thus, Reimer (144) has clarified the analysis of percentage data, and Healy (in 131) has explained the general nature of statistical argument in simple terms and summarised some of the basic methods useful in insect work.

There is no excuse nowadays for inadequate analysis; but there are still many cases of inappropriate analysis, partly because statisticians give precise shades of meaning to words that many entomologists interpret in a much looser sense. A common language will doubtless develop in time because of the unquestioned value of a mathematical approach to biological problems in general. In this context, the current arguments about insect population growth and the rights and wrongs of mathematical modelism are of great value; if they do nothing else, they certainly direct attention to underlying assumptions and interpretations and encourage habits of thought which are already producing dividends in a generally more critical approach to field problems.

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# BIOLOGY OF CHIGGERS[1]

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Chiggers are the common name for members of the mite family Trombiculidae Ewing, 1944, and are also called *tsutsugamushi* or *kedani* in Japan, or harvest mites in Europe. They have attracted special attention, not only because a number of species attack humans and cause dermatitis, but also because several species transmit a rickettsial disease called "scrub typhus" or "tsutsugamushi disease" in the Oriental and the Pacific Regions.

Remarkable advances have recently been achieved in studies on the mites and the disease, and the prewar concepts have been enlarged upon or modified. Taking the taxonomic status of the mite family Trombiculidae as an example, the numbers of species, subgenera, and genera known to the world have greatly increased after World War II. Linnaeus (1758) included in his *Systema Naturae* only a single species of the chigger. Three species had been described prior to 1900. In 1912, Oudemans (74) listed 27 species in his monograph. Some 100 species had been recorded by 1942. Wharton & Fuller (111) in 1952 listed 440 species in their monograph of chiggers. The number of chigger species discovered in various regions of the world increased year by year and was roughly estimated to be over 1000 in 1959, or about one-tenth the total number of recorded species of the mite or Acarina, though chiggers comprise only a single family among some 200 families of the order Acarina.

## Life History

*Developmental cycle.*—A chigger passes through four developmental stages in its life: egg, larva, nymph, and adult. An ecdysis takes place at the transformation from one stage to the next. So far as is known, eggs of chiggers are all globular when laid by the mother mite; in about a week, the eggshell splits into two hemispheres and a maturing larva, or the deutovum, emerges. A larva is six-legged, and only one-fourth to one-half of a millimeter in body length when unengorged; the larval stage is the only parasitic one, at which time the larva attaches to a vertebrate (rarely arthropod) host until it is fully engorged with the tissue fluid; it then leaves the host and passes a quiescent stage called nymphochrysalis, or prenymph. Nymphs and adults are eight-legged and free-living. A nymph passes the quiescent stage called imagochrysalis, or pre-adult, before molting into an adult.

The outline of the generalized life cycle of chiggers presented above was elucidated as early as 1916 by Nagayo *et al.* (70), Miyajima & Okumura (66),

[1] The survey of the literature pertaining to this review was concluded in January, 1960.

and Kawamura & Yamaguchi (44) through field and laboratory observations with *Trombicula* (*Leptotrombidium*) *akamushi* (Brumpt), the vector of tsutsugamushi disease in northwest Japan. Rearing of chiggers from larvae to adults was later reported by Ewing (17) with *Hannemania hylae* (Ewing) and *Trombicula alfreddugèsi* (Oudemans), by Melvin (62) with *Trombicula batatas* (Linnaeus), and by Michener (64, 65) with the above and two other species.

It was after 1946, however, that observations on the complete life cycle of chiggers from larva to the next generation larvae became feasible in the laboratories. In that year, Wharton & Carver (110) and Jayewickreme & Niles (33) independently discovered that nymphs and adults of some chiggers could successfully be reared by supplying insect eggs for food; their real food habit had so far remained unknown, and the laboratory rearing of the postlarval stages had been only partially successful. By adopting this technique, Wharton (109) succeeded in breeding *Euschöngastia indica* (Hirst), and Jayewickreme & Niles (33) obtained adults of *Trombicula acuscutellaris* Walch. Observations on the complete life history of chiggers were later reported by Jenkins (34) for three species of the North American chiggers, by Cockings (12) for *Trombicula deliensis* Walch, by Jones (39, 40) for *Trombicula autumnalis* (Shaw), and by Sasa *et al.* (89) for *T. akamuski* (Brumpt). Further reports on the life history of the above and some additional species have been made by Wharton & Fuller (111), Sasa and coworkers (83, 87, 88, 91), Audy (7), Lipovsky (56, 57), Wolfenbarger (113), Farrel (19), and Ito *et al.* (32). The behavior of the mite in each stage or at metamorphoses was recorded in a motion picture film by Sasa & Miura (86).

The mode of insemination of chiggers, which had long remained unknown, was recently demonstrated by Lipovsky *et al.* (58) for several trombiculid species. They observed that females were inseminated, not by direct copulation, but by means of spermatophores deposited by the male on the substrate. The spermatophores described for four species consisted of a flexible stalk attached to the substrate and a sack of sperm at its distal end. Females that were isolated from males but exposed to the spermatophores deposited fertile eggs, and there was no evidence for parthenogenesis.

*Developmental times and generations.*—The times required for chiggers to develop differ greatly by environmental conditions, such as temperature or the kind and amount of food supply. The above-mentioned authors (7, 12, 19, 32, 33, 34, 39, 40, 56, 57, 83, 87, 88, 89, 91, 109, 110, 111, 113) reported on the times for developments under various conditions. For example, Sasa & Miura (87) obtained the following results with laboratory rearings of *T. akamushi* under 28°C., giving eggs of *Culex pipiens* Linnaeus as food for postlarval stages: an average of 7.3 days for active engorged larvae, 8.3 days for prenymphs, 25.3 days for active nymphs, 8.3 days for pre-adult, 29.3 days from emergence of adults to the first oviposition, 14 days for the egg stage, and 2 days for engorgement of larvae on rats, with a total of about 75 days for completing the life cycle. Under favorable laboratory conditions,

the time required for completing a life cycle was reported to be about 60 days for *E. indica* by Wharton (109), 40 days for *T. deliensis* by Audy (7), and 50 to 70 days for the common North American chiggers by Jenkins (34).

The number of generations a year for chiggers in nature also differs greatly by local climate and by species. Most chiggers in the temperate zone, such as *Trombicula* (*Leptotrombidium*) *scutellaris* Nagoyo *et al.* and *T. akamushi* in Japan or *T. autumnalis* in Europe, seem to have only a single generation per year (see section on seasonal distribution). According to the review by Wharton & Fuller (111), *T. alfreddugèsi* produces one or two generations a year in Ohio, three generations in North Carolina, and the production is continuous in Florida. Audy (7) suggests that *T. deliensis* produces at least three generations a year in the Indo-Burma region and five or more in Malaya.

## Laboratory Culture

Attempts to rear chiggers have been made by a number of workers for various purposes since the beginning of this century, but successful results in breeding chiggers for generations have been only recently achieved. This is obviously attributable to the discovery of the food habits of the postlarval stages, improvements in the substratum and containers, and also to the increased information on the behavior of unengorged larvae. At present, chiggers are kept in several institutions throughout the world as laboratory colonies. They are usually started with engorged larvae collected in nature, which spontaneously develop into nymphs on charcoal-gypsum substratum under conditions of adequate temperature and humidity. Insect eggs are given as food to nymphs and adults. Eggs are laid on the substratum, from which the larvae hatch. They are attached to laboratory animals and are recovered after engorgement.

*Collection of materials.*—The source of materials to begin breeding is usually engorged larvae that have detached themselves from a host. Small mammals (especially rodents) trapped in the field often habor large numbers of chiggers, but wild birds, reptiles, or amphibians are sometimes the important hosts for special species of the mites. The engorged larvae may be collected from hosts with a moistened brush or a needle, but it is more efficient to use the "hanging method" introduced by Teramura (104) or the "washing method" reported by Lipovsky (54). In the former method, a small mammal is tied by one of its legs and hung from a bar above a petri dish containing water; ectoparasites, including chiggers, detach themselves from the host within one or two days and can be recovered by a brush from the surface of the water in the dish. The latter method consists of washing the ectoparasites off a host with water that contains detergent and then recovering them from the sediment. Chiggers may be preserved alive on a host for months when kept in a freezer.

For the recovery of nymphs and adults from the soil, the "flotation method" introduced by the classical Japanese workers and described by

Kawamura & Ikeda (43) still has many advantages. A sample of soil is broken apart in a large container of water; many arthropods, including all stages of chiggers, are collected as they come to the surface on bubbles. Recent reports on the application of this method were made by Cockings (12), Sasa (83), and Loomis (59). Unengorged larvae may also be recovered from the soil by various methods, as will be discussed later.

*Containers.*—Various types of glass containers with tightly closed caps have been used by most workers; for example, Wharton & Fuller (111) recommended wide-mouthed canning jars, and Lipovsky (56) reported that chiggers could successfully be shipped or reared in a 3- or 5-dram plastic-stoppered vial lined completely with a charcoal and plaster of Paris mixture. Sasa & Miura (88) have described their containers for mass and individual rearings and stated that better results were obtained with plastic dishes that were 6 to 9 cm. in diameter and 2 to 3 cm. in height and tightly stoppered by a filter paper sheet inserted between the cover and the dish; such types of plastic containers could more conveniently be examined under the microscope and harbored less danger of mites being trapped in water droplets than in the glass containers.

*Substratum.*—After Wharton (109) used the mixture of one part of activated charcoal and nine parts of gypsum (plaster of Paris), it was universally adopted as the substratum for all stages of chiggers. A paste is prepared by adding water to the mixture and is poured into a container so as to form a solid flat layer of at least 1 cm. thick. It also serves as the humidifier. Farrel & Wharton (20) reported that the addition of a layer of vermiculite to the surface of the substratum gave better results in the mass breedings. It is necessary to recover the cast larval skin to identify the species of unknown nymphs or adults. Special devices for their individual rearings have also been reported (88).

*Rearing of postlarval stages.*—Postlarval chiggers, except those in quiescent stages, require some small arthropods or their eggs as food. The failures of many earlier workers to breed chiggers for generations were mainly attributable to lack of information on this food habit. Wharton (109) and Wharton & Carver (110), working with *E. indica*, discovered that eggs of *Culex* and *Aedes* mosquitoes, *Drosophila*, etc., served as food for nymphs and adults; these authors also described their feeding behavior. Jayewickreme & Niles (33) fed *T. acuscutellaris* and *T. deliensis* with eggs of *Culex fatigans* Wiedemann. Sasa and co-workers (87, 89) reared *T. akamushi* and several other species with eggs of *Culex pipiens pallens* Coquillet. Hyland (31) fed *Trombicula splendens* Ewing with eggs of *Anopheles quadrimaculatus* Say. Lipovsky (55) observed that collembolans and their eggs serve as food and introduced a convenient method of breeding postlarval chiggers and collembolans in the same containers by giving brewers' yeast as food for the latter. Ito *et al.* (32) reported on the excellent results of breeding *T. akamushi* with eggs of dragonflies. An extensive study on the food habit was made by Lipovsky (57) with *T. alfreddugèsi* and *T. splendens*; he observed that some

of the arthropod eggs were repellent, toxic, or unattractive to the post-larval chiggers, while those of some Collembola, Diptera, Hemiptera, and Lepidoptera served as adequate foods. Morishita & Nakamatsu (69) observed that *Trombicula fuji* Kuwata *et al.* fed on the soil nematode, *Rhabditis hominis* Kobayashi.

*Rearing and preservation of larvae.*—Female mites deposit eggs on the substrate in containers to which adequate food is supplied under favorable temperatures (25° to 32°C.). After about two weeks of incubation, larvae hatch from the eggs. Sasa & Miura (88) reported that unengorged larvae of *T. akamushi* or *T. scutellaris* could be preserved for several weeks on a charcoal-gypsum cone in a moist container.

The larvae must feed upon an adequate host before they can pass to the next stage. Various methods have been reported to recover engorged larvae. Most workers keep a host in a small cage over a funnel with one or two layers of screen in the bottom. Engorged larvae detach from the host, crawl down through the screen and drop onto the water surface under the funnel. Michener (64) used chickens and Jayewickreme & Niles (33) used laboratory mice as hosts. Jenkins (34) recommended snakes and turtles as hosts for *T. alfreddugèsi* and *T. splendens*, because they could easily be handled and caused little contamination by feces or food which otherwise often trapped the mites. However, the reptiles serve as hosts only for restricted species of chiggers, such as the members of the subgenus *Eutrombicula*, and most of the other important groups require mammals or birds to engorge.

The rate of recovery of engorged larvae is usually small when laboratory mice or rats are used, mainly because they scratch off the mites before attachment. Ito *et al.* (32) obtained better results by immobilizing legs of laboratory rats used as experimental hosts. Wharton & Fuller (111) recommended young hamsters for this purpose. Tanaka *et al.* (100) reported that better recovery was obtained by using *Microtus montebelli* (Sado) (Japanese field vole) as the experimental host for *T. scutellaris* than by using mice, rats, or gerbiles.

## Behavior of Unengorged Larvae

From the standpoint of public health, the behavior of unengorged larvae waiting for the opportunity to bite a host is the most important aspect in the bionomics of chiggers. Information pertaining to this subject has been accumulated rather slowly, simply because they are extremely minute creatures difficult to detect by the naked eye. However, various methods for recovering the mites from the soil have been developed, and, consequently, the analytical studies on their behaviors have recently made remarkable progress.

*Collecting methods.*—In certain areas where there is a large population of those species that have the habit of congregating into clusters, chiggers may be collected by visual examination, as reported for *T. autumnalis* by André (1) or for *T. scutellaris* of Hachijo Island, Japan, by Ueno *et al.* (107)

and Sasa *et al.* (90). However, such instances were rather exceptional, and chigger workers had to apply various indirect collecting methods.

(*a*) *Plate method.*—It is known that some chigger larvae can be captured by placing various objects on the infested ground. One of the earliest observations was that reported by McCulloch (60), who recovered the mites from the boots of investigators. Smith & Gouck (95) utilized pieces of black oilcloth 12 inches square for evaluating the effects of miticides on chiggers. Williams (112) used white china saucers for collecting the North American chigger, *T. alfreddugèsi;* according to Wharton & Fuller (111), this constituted the same method as had been used at Duke University for about 10 years. Linduska *et al.* (53) tested the effects of control procedures on chiggers by setting out black cards, and Gasser & Wyniger (23) used flags. Later investigators have mostly replaced the white dishes, cards, or cloth by black plastic or bakelite plates of several inches square, since they are easier to be handled, more attractive to the chiggers, and more closely in contact with soil surface [Jenkins (36); Kardos (41); Loomis (59); Michener (65); Suzuki and co-workers (96, 97, 98); Wharton & Fuller (111)].

(*b*) *Light trap method.*—Cockings (12) and Jones (38) constructed field light traps that were efficient in capturing large numbers of the European harvest mite, *T. autumnalis*, for which the plate methods were less effective. They utilized the method for assessing the population and the mode of distribution of the mites in the infested areas. Kumada (49), Sasa (83), and their colleagues employed light traps of much simpler structure in the early stages of their field studies on *T. akamushi*, but these were later replaced by the plate methods because the latter were found to be more efficient in collecting this species.

(*c*) *Berlese funnel.*—For the chigger species that are difficult to recover by the above methods, the Berlese funnel, widely used by the acarologists to recover soil mites, may be useful. Wharton (109), Farrel (19), and Kepka (46) successfully collected larvae of *Euschöngastia* from soil and other materials. However, the method may be again unsuited for other species, as reported by Richards (80) for *T. autumnalis*.

*The rate of movement.*—Although chiggers are extremely minute creatures, their movement is very fast. Their speed is greatly influenced by temperature and substrate and also differs by species. Jenkins (36) measured the movement rate of *T. alfreddugèsi* and *T. splendens* on a horizontal glass surface at various temperatures and humidity and found that the relation of movement with temperature fitted a straight line between 18° and 36°C., with a rate of 24 cm. per min. at 25°C. and $Q_{10}$ of 2.15. The rates obtained by Kohls *et al.* (48) for *Trombicula hirsti* Sambon were on a similar level when tested on a flat surface, though their data varied greatly with the substrate.

Wharton & Fuller (111) and Farrel (19) compared the rate of locomotion of *T. alfreddugèsi* and *Euschöngastia peromysci* (Ewing) and found that the latter was active to temperatures below freezing, whereas the former

became immobile at +10°C. The rates observed for *Trombicula wichmanni* (Oudemans) by Hayashi *et al.* (30) and Sasa *et al.* (92) averaged 22.3 cm. at 25°C. and 54.8 cm. at 34°C. The above four species are all long-legged and closely related, being of the same subgenus *Eutrombicula*, and were found to move at a similar speed. Sasa *et al.* (92) reported that *Neoschöngastia* spp. also crawled as fast as the above species. On the other hand, the movement rates observed by Sasa *et al.* (92) for *Trombicula* (*Toritrombicula*) *hasegawai* Sasa *et al.* and by Suzuki (96, 97) and Hayashi *et al.* (30) for *T.* (*L.*) *akamushi* and *T.* (*L.*) *scutellaris* were much smaller, being only about one-fourth of the former group. The discrepancy between these two taxonomic groups seems to be associated with the difference in the biological attitude of attaching to the hosts.

*Reaction to stimuli.*—As was recently mentioned by Wharton & Fuller (111) for chiggers and by Dethier (14) for blood-sucking arthropods in general, only a few species of trombiculid mites have been the subject of intensive investigations for their sensory physiology, and little is known about the significance of the reaction of unattached larvae in nature to various physical or chemical changes in the environment. Jenkins (36) observed that the activity of unengorged larvae of *T. splendens* and *T. alfreddugèsi* was initiated by changes in intensity or direction of light or by movement of air, of the substrate, or of a nearby object. The direction of movement was determined almost entirely by light, being positive if the larvae had been adapted to higher light intensities and negative if adapted to lower intensities. Gravity had no effect in determining the direction of movement. Larval movement was stopped by sudden changes in light, temperature, sound, or air current. Cockings (12) and Jones (38) each designed a light trap and demonstrated that unattached larvae of *T. autumnalis* were attracted to the bright area. Jones (37) also observed that sudden reduction in the intensity of light elicited a "questing response," which consisted of waving the anterior legs in the air and at times resting the body on the substrate and elevating all of the legs. He also reported an absence of definite geotaxis, but noticed that mites placed on a vertical rod tended to move upward during the first five minutes. As for the chemoreception, Dethier (14) stated that there were no specific stimuli known to cause the larvae to search actively for a host.

However, a series of investigations recently made by Sasa and co-workers with various species of the Japanese chiggers have solved a part of the riddle of their behavior in nature. The observation was started with the discovery by Ueno *et al.* (107) of the fact that unengorged larvae of *T. scutellaris* in Hachijo Island usually concealed themselves in shaded niches, like small holes in the rocks, but appeared on the surface a few seconds after an observer approached the objects, and crawled to the top to form clusters of vigorously moving mites. The important point of this observation ("Ueno's phenomenon") was that the mites were sensitive to the approach of a possible host. In the second step of the studies reported by Sasa *et al.* (90) and

Tanaka *et al.* (101), the mites were brought into the laboratory and observations were made to see the behavior under various environments. It was soon recognized that when the mites were placed on the surface of a small cone made of the gypsum-charcoal mixture and were kept in a glass container, they crawled around for a while but gradually climbed up toward the top and formed a cluster on the shaded side. Under such a condition, movement of the mites was apparently directed by the two components, negative geotaxis and negative phototaxis, and, thus, they gradually aggregated in a small area of the cone near the top and opposite the window. The third character detected here was a kind of thigmotaxis, by which the mites remained still in a cluster on the exposed surface when their bodies were put in contact with each other. As long as the mites on a cone were kept in a moist and compact chamber, they never moved again and could be preserved alive for several months.

The third step of this study was to unveil the nature of the stimulus that caused the motion of these mites. Various physical and chemical effects that might be caused by the approach of a host were tested against the mites resting in a chamber. Sound, vibration, heat, air current, and changes in light and humidity were all ineffective. Among the volatile chemicals tested, hydrochloric, acetic, lactic and butilic acids, ethyl- and methylalcohol, or acetone never acted as the physiological stimuli, though they might have caused a type of avoiding reaction. Likewise, human sweat or saliva did not affect the mites. The only factor to which the mites reacted was the air current containing human or animal breath.

The next step was analysis of the breath to detect the active components. It soon became clear that the effective component was an acid substance, since the breath throughly washed with alkali solutions completely lost its effect, whereas that treated with acid or organic solvents remained effective. On the other hand, carbon dioxide gas chemically prepared and diluted with the air to extremely low concentrations was found to cause the immediate reaction of the mites in the same manner as did the breath. Through these experiments, Sasa *et al.* (90) concluded that unengorged larvae of *T. scutellaris* in nature originally stayed still in a shaded niche near the top of various objects on the earth, thus protecting themselves from consumption and dessication, and when a possible host approached their environment they recognized it by an increase in the carbon dioxide in the air and immediately exposed themselves near the top of the object so as to grasp the opportunity of attachment.

The same group of workers (90) later demonstrated that unengorged larvae of *T. akamushi* had the same characteristics of cluster formation and response to carbon dioxide. Sasa *et al.* (92) further detected that larvae of *T. wichmanni*, *T. hasegawai*, and *Neoschöngastia* spp. reacted to carbon dioxide as well, though they differed from the above two *Leptotrombidium* species in that they never formed a mite cluster on an exposed surface but had the habit of staying motionless individually in small holes; this was an

adaptation to their season of appearance, summer, and their habitats, which are rather dry.

The characteristic of noting the approach of a vertebrate host by carbon dioxide seems to be widely shared by parasitic mites and insects. Sasa & Wakasugi (93) reported on the stimulating effect of the gas on the tropical rat mite, *Ornithonyssus bacoti* (Hirst), and Sasa (84) on the fleas, *Ctenocephalides canis* (Curtis) *Monopsyllus anisus* (Rothschild) and *Nosopsyllus fasciatus* (Bosc). Sasa & Shirasaka (unpublished) further detected that infective larvae of human or animal nematode parasites, *Ancylostoma duodenale* (Dubini), *Ancylostoma caninum* (Ercolani), *Necator americanus* (Stiles), and *Trichostrongylus orientalis* Jimbo, also reacted to carbon dioxide and to breath. In all of these cases, the gas acted as a stimulant to release a motion of the parasites that were in a resting stage and not as an attractant to determine the direction of their movements. In the chiggers exclusively parasitic on birds, such as *T. hasagawai* and *Neoschöngastia* spp., Sasa *et al.* (92) reported that the breath of either birds, humans, or rats acted equally well, but the birds had an unknown factor, which was absent in mammals, that attracted these mites.

As was reviewed by Dethier (14), several North American workers reported on the effects of carbon dioxide on mosquitoes. Similar results were also obtained by Osada (73) for some Japanese mosquitoes. In all these observations, the mosquitoes seemed to utilize the gas as an attractant to approach a host and not as the stimulant to provoke the flight, thus differing essentially from the above-discussed arthropods.

It should also be noted here that the behavior of these parasitic arthropods, including chiggers, are not the same when they are in a quiescent or tranquilized state isolated from an observer's breath as when in an agitated stage under direct influence of waves of carbon dioxide. The discrepancy between the results of different workers in the sensory physiology of these parasites, as pointed out in the review by Dethier (14), seems to originate from the experimental conditions and whether the workers exposed the test animals to their own breath.

## Spatial Distribution

The mode of distribution of chiggers in space is discussed here under the categories of the geographical, topographical, surface, and vertical distributions. The first three categories are tentatively defined by the size of the areas to be considered. The geographical distribution seems to be limited mainly by the evolutionary origin, followed by geographical segregation; the mode of local or topographical distribution is mainly determined by the ecological environments; the distribution of individual chiggers within a small area of ground surface is apparently determined by the physiological characters of each species.

*Geographical distribution.*—The mite family Trombiculidae is almost cosmopolitan, and species have been found on practically every land mass in-

vestigated. However, there are great gaps in our knowledge of the trombiculid fauna of the world, and only a few localities, such as a part of Europe, the United States, Malaya, and Japan, have been thoroughly investigated. Wharton & Fuller (111) gave an account of the geographical distribution of some 440 species recorded prior to 1951, but the number has been doubled by the elapse of several years.

In spite of the gaps in our present information on the world trombiculid fauna, some characteristics in the geographical distribution may be pointed out. With the exception of a few genera, such as *Trombicula* or *Euschöngastia*, which are cosmopolitan and are represented by numerous species in many parts of the world, most groups (genera or subgenera) are restricted or concentrated in their distribution to certain regions of the world. Wharton & Fuller (111) concluded that trombiculids as a whole are best represented in the region of Australia, southern Asia, and Africa and the adjacent islands, with a few exceptions, such as *Apolonia* and *Womersia*, which are confined to America. Audy (7) suggested that such genera as *Gahrliepia*, *Walchiella* and *Traubacarus* evolved in the tropical forests, while the subgenus *Leptotrombidium*, including the main vectors of scrub typhus, had its cradle of evolution in the Palaearctic rather than Oriental Region.

The distribution of trombiculid species or minor species groups are usually extremely restricted, especially for those parasitic on small mammals, though some species parasitic on birds, such as some *Neoschöngastia* or certain *Trombicula*, may be common to both Asia and America. Brennan & Wharton (10) have shown that the subenus *Neotrobicula* in North America may be classified into three groups by taxonomic characters and by geographical and seasonal distributions, and a similar pattern is found in other trombiculids, with few exceptions.

Sasa (83), in his monograph on the Japanese trombiculids, discussed the nature of the geographical distribution of this mite group. Of some 60 species described from this country, only 17 are known to occur abroad. The distribution is more or less restricted, even within this small country, and only three species are known to occur on all of the four main islands. Morphological variations of subspecific nature, closely associated with the localities of collection, were often encountered in species with wide ranges of distribution. Several species are found only in the western parts of Japan, and the limiting line can be drawn across central Honshu from north to south. Sasa suggested that the geographical segregation is more important in limiting their distribution than is climate. Species like *T. hasegawai* are extremely restricted in their occurrence, having been collected only from Hachijo Island. Such species as *T. akamushi*, *T. scutellaris*, and *Trombicula tosa* Sasa & Kawishima (all known or suspected vectors) were sporadic in their mode of distribution, not being continuous and widespread like *T. fuji* or *Trombicula pallida* Nagoyo *et al.*

*Topographical distribution.*—The distribution of a chigger species is generally habitat specific; usually one or more species of major hosts occupying a particular niche are attacked. Furthermore, chiggers often show

habitat preference within the home range of their hosts. The mode of local distribution of a chigger species may also differ by environment (such as type of soil, vegetation, or climate), as will be discussed in the section on epidemiology.

A number of chigger workers have demonstrated the specific habitats of various species and their hosts. The most remarkable example is seen in distribution of *T. akamushi* in Japan; this species is restricted to the flooded zones along the four rivers of northwestern Honshu, which is inhabited by a large number of field voles, whereas several other species occur both in and outside of such endemic foci. In Malaya, Audy (7), Audy & Harrison (8), and Harrison (26) reported that *T. akamushi* was the predominant chigger in the grassland, with field rats as the host, whereas *T. deliensis* was more abundant on wood rats in the oil palm estates; they also stated that the forest floor was the only habitat for other species of *Leptotrombidium*, and *Euschöngastia* was the only chigger found on tree squirrels in the forest canopy. Brennan & Wharton (10), Wharton & Fuller (111), Loomis (59), Wolfenbarger (113), and Kardos (41) in North America, Kepka (46) in Europe, and Sasa (83) in Japan have also presented informations in this line.

Although most chiggers breed in the soil of grass or forest areas and are usually more abundant along the forest edge, some species are known to have peculiar niches. Wharton (109) reported that *E. indica* in Guam were found in greatest numbers in the rhizomes of epiphytic ferns. Wharton & Fuller (111) summarized information accumulated by various authors on the unusual habitats of chiggers, such as those found associated with ant nests, on water lettuce, or in holes of logs. Sasa *et al.* (91) reported that some species of *Neoschöngastia* breed on rocks along the sea shore.

*Ground surface distribution.*—As a result of the recent advances in collecting methods of individual chiggers from the ground surface, the mode of distribution on or in the soil has gradually been elucidated for a number of epidemiologically important species. Cockings (12) and Jones (38) reported that the larvae of *T. autumnalis*, which they collected with light traps from small areas, were extremely concentrated to some shaded, moist foci or the "pocket of infection." Wharton & Fuller (111) made a survey of chiggers in a forest with the disc sampling method and found that among various soil and cover types the combination of sand and thickets was most suited as the habitat for *T. alfreddugèsi* and also that soil moisture was the main factor in segregating this species from *T. splendens*, which was abundant only where it was wetter.

Suzuki (96, 97), in his studies on the control of chiggers with miticides, applied the bakelite plate method to recover unengorged larvae of *T. akamushi* and *T. scutellaris* from the ground surface; he also found that they were extremely concentrated in moist shaded areas in the endemic foci of scrub typhus. He further demonstrated that the mite counts within small areas of 5 m. or 1 m. square were not distributed evenly by chance but showed patchy distributions. Hayashi *et al.* (30) made further analytic-statistical studies on the mite counts in small areas infested by either *T.*

*akamushi*, *T. scutellaris*, or *T. wichmanni* and found that the distribution of the former two species did not follow the Poisson law as expected by chance, but that the last species was almost evenly distributed on the ground surface. The discrepancy between the mode of ground surface distribution of the above two groups, the former being the subgenus *Leptotrombidium* and the latter *Eutrombicula*, is probably associated with the difference in the behavior of the unengorged larvae. As is discussed in the previous sections of this review, the former is long-legged and fast-moving, whereas the latter is short-legged and rather sluggish in its movements; furthermore, the latter group of chiggers tend to form compact mite clusters on the ground surface while the former usually remains alone without forming the cluster.

Techniques other than the light trap or the plate methods were also applied to estimate the mode of surface distribution of chiggers. Farrel (19) and Kepka (46) used the Berlese funnel method for collecting *Euschöngastia* spp. from the soil and discussed their mode of distribution in relation to the type of vegetation. Tanaka *et al.* (103) also demonstrated the clumped distribution of *T. akamushi* by counts on field voles in relation to the home range of the hosts. Sasa *et al.* (91, 92) reported that some of the chiggers of Hachijo and Miyake Island could be recovered from the surface of rocks by applying the observer's breath, and they utilized this method in counting the number of mites present on each rock of an area. They found that chiggers on rocks were not evenly distributed on the surface but were concentrated near the top, and the numbers recovered from individual rocks did not follow Poisson's law.

*Vertical distribution.*—Most chiggers are soil dwellers, except in the parasitic period, but little has been known about their vertical distribution in the soil. Kawamura & Ikeda (43) collected adults of *T. akamushi* from soil samples of various depths with their flotation method and found that they were most abundant in the range of 3 cm. from the surface but could be recovered up to 18 cm. They also observed that the mode of distribution differed by season, being deeper as the soil temperature dropped. Suzuki (96) reported on his results of recovering unengorged larvae of the same species with the bakelite plates set at various depths and stated that they were almost exclusively distributed on the exposed surface, a few within 3 cm. depth, and none below 5 cm. The vertical distribution of adults of *T. autumnalis* was observed by Cockings (12). He concluded that the adults went deeper as the temperature dropped near freezing or as the soil became too dry or too wet, in which case they were concentrated at a depth between 12 and 18 inches. Kepka (46) investigated the vertical distribution of the larvae of *Euschöngastia xerothermobia* (Willmann) with the Berlese funnel method and found that they migrate vertically by season.

## Chronological Distribution

*Diurnal distribution.*—The activity of chiggers, especially of unengorged larvae, is much influenced by the meterological and climatic conditions,

which cause various types of dirunal or seasonal rhythms in their population or activity. André (1) observed that larvae of *T. autumnalis* were scarce in the morning, became numerous in the afternoon, clustered in groups toward evening, and resumed activity the next morning. Richards (80), Tuxen (106), and Gasser & Wyniger (23) also observed the dirunal activity of this species. More detailed study along this line was reported by Wharton & Fuller (111) for *T. alfreddugèsi*. The number of mites collected with the disc sampling method was recorded every hour for 62 consecutive hours in three stations. On the ground chiggers were most active in the daytime between 7 a.m. and 7 p.m. under all types of weather conditions. On rock, however, a drop in the mite count was observed between 11 a.m. and 5 p.m. as the rock became very hot when exposed to the noonday sun. They concluded that temperature was the main determining factor in the diurnal activity of the chiggers.

Some observations were also made on vectors of scrub typhus. It has long been the experience of people in the classical endemic foci of the disease in Japan that the attack of *T. akamushi* takes place oftener in the morning when it is cool and the soil is wet than in the hot and dry midday hours. This was also confirmed by Audy (7) for the vectors in Malaya. Kohls *et al.* (48) in New Guinea found that the pest chiggers were common on boots both during the day and at night. Suzuki (96) in northern Japan also stated that no significant difference was seen in the diurnal activity of *T. akamushi*, as estimated from counts on bakelite plates. However, Suzuki and co-workers (98, 99) later observed that *T. akamushi* in the same locality were more active in the daytime than at night with the highest mite count in the midday, whereas *T. scutellaris* in winter on Hachijo Island showed two peaks in its counts, in the morning and in the afternoon. The discrepancy between these results suggest that diurnal activity of chiggers is not dependent on chronological factors but is mainly determined by diurnal changes in microclimate, such as temperature or humidity.

*Seasonal distribution.*—Most chiggers, especially the larval stage, exhibit remarkable seasonal fluctuation of the population in nature. In the temperate zone, temperature seems to act as the main determining factor, whereas the amount of precipitation is a more important cause in most tropical regions. The activity of larval chiggers may differ by species; some are commonly called summer or winter chiggers, or the harvest mites.

In Europe, where the chigger fauna is rather simple, a number of workers investigated the seasonal distribution of the larvae of the main pest chigger or the harvest mite, *T. autumnalis* [Cockings (12); Daniel (13); Elton & Keay (15); Jones (38); Keay (45); Kirshenblat (47); Oudemans (74); Pyontovskaya *et al.* (78); Richards (80)]. The activity of the larvae of this species seemed to be at its peak in the summer toward autumn and is confined to the months of August and September in northern regions like England but is more delayed and prolonged in the south, such as in the Caucasus or Czechoslovakia. As for the other species, the only information avail-

able was that reported by Vysotskaya & Schluger (108) in the Leningrad area and by Daniel (13) in Czechoslovakia for *Trombicula zachvatkini* Schulger. The species was found to be a type of winter chigger with two to several peaks, as influenced by meteorological conditions. Recently, Kepka (46) reported on the seasonal distribution of *E. xerothermobia* in Austria in soil samples and found that the larvae were more abundant during the period from September to May than in the summer.

In North America, the activity of the common pest chiggers (*T. alfreddugèsi* and *T. splendens*) is also confined to the warmer season of the year, from about two months in the northern States to the entire year in southern Florida [Ewing (16); Jenkins (35); Loomis (59); Wharton & Fuller (111)]. Wharton & Fuller (111) and Loomis (59) studied the correlation between the climate and the seasonal appearance of the chiggers and found that temperature, not the amount of rainfall, was the main limiting factor. Gould (24) observed that the pest chigger in California, *Trombicula belkini* Gould, reached its peak in late summer or fall. Brennan & Wharton (10) classified the North American species of the subgenus *Neotrombicula* into the following three groups: the northern group, which appears in the summer; the southern group, which appears in the winter; and the western group, which appears throughout the year. Wharton & Fuller (111) stated that chigger species in other taxonomic groups might fall into one or another of the above geographic-seasonal groups or into the fourth group represented by the common pest chiggers, which were widely distributed and occurred only in the warmer months of the year. Additional information pertaining to the North American chiggers was assembled by Loomis (59) in Kansas, Gould (24) in California, Kardos (41) for *Neotrombicula*, and by Farrel (19) for *Euschöngastia*.

In Japan, the seasonal distribution of the main species of chiggers has been extensively surveyed by a number of workers in different regions. The earliest contributions were made by Nagayo *et al.* (71) in Yamagata and by Kawamura (42) in Niigata. Kawamura & Ikeda (43) studied the seasonal occurrence of larval chiggers on the voles in the endemic foci of tsutsugamushi disease. They found that *T. akamushi* was the only species that occurred predominantly in the summer while the disease was prevalent, whereas several other species were present only during other seasons of the year, in autumn or from autumn to spring. Teramura (104) in Akita was the earliest postwar worker who resumed study in this line, and he found that the chigger fauna was entirely different in the endemic foci in the alluvial flats of the river than in the nearby forest outside the banks and that the seasonal distribution of chiggers on field voles and mice differed remarkably by species.

Following the recent discovery by the Japanese workers that the occurrence of chiggers was not restricted to the classical endemic foci of scrub typhus but almost all over the country, observations on the seasonal distribution have been made at about 60 stations distributed from Hokkaido

to Kyushu. Between 1952 and 1959 a total of about 50 papers dealt with the seasonal distribution of the Japanese chiggers, and the authors involved exceeded 100. The seasonal distribution of chiggers in Korea was surveyed by Chun & Chung (11).

Sasa (83), in his monograph of the Japanese chiggers, reviewed the results obtained by these workers and proposed the idea that chiggers could be classified into several groups by the biological characters of the seasonal distribution, such as the summer, autumn, winter, or autumn-spring group. Temperature is obviously the main determining factor for the appearance or activity of larvae; each species has the optimum average temperature range for its larval activity, such as over 20°C. for certain summer group members, between 20°C. and 10°C. for certain autumn groups, or under 20°C. for some winter or autumn-spring species. Consequently, the period of appearance of the same species may differ by the local climatic conditions; the summer group, such as *T. akamushi*, appears earlier and is active for longer periods at localities toward the south, whereas the autumn group such as *T. scutellaris* is delayed in its larval appearance but prolonged in its period of activity toward the south. The same range of temperature might affect in an entirely different way the appearance of larvae (i.e., oviposition of adults), whether it is in autumn (declining temperature) or in spring (rising temperature). Most of the chiggers in Japan and in Korea (especially *Leptotrombidium*) are the autumn-spring group, having two peaks in autumn and spring and being almost or entirely absent in the summer; groups like *T. akamushi* and *T. tosa* (summer) or *T. scutellaris* (autumn) are rather exceptional members of *Leptotrombidium*, though they are epidemiologically important.

In the tropical zone, the seasonal activity of chiggers may primarily be determined by the precipitation, in contrast to the above-mentioned fact that temperature plays the main role in most of the temperate regions. Audy (7) has summarized the information he and his co-workers and Traub (105) accumulated in South Asia; in India and Burma, there is a typhus season during the monsoon climate from July to November, the onset of the rains is followed by a sudden increase in chiggers, which pass two or three generations in the hot-wet season, and egg-laying apparently ceases for some months through the dry season; in Malaya and Sumatra, however, there is no such seasonal interruption, and the chigger populations remain high throughout the year except for minor fluctuations because of drier or wetter periods.

## Host Relationship

*Host specificity.*—Chiggers or members of the family Trombiculidae are characterized by taking vertebrates as hosts for the larval stage; a few exceptional species have been found on arthropods. All classes of terrestial (and a few aquatic) vertebrates have been known to serve as hosts. Harrison & Audy (28, 29), Wharton & Fuller (111), and Radford (79) compiled host

lists of the world species, but a large number of additional records were later published by various authors.

It has been a well-established fact that certain species (or groups) of chiggers prefer certain species (or groups) of vertebrates as hosts; for example, species of *Hannemania* occur exclusively on amphibians, *Neoschöngastia* on birds, some of *Eutrombicula* on reptiles, and *Leptotrombidium*, *Neotrombicula*, *Miyatrombicula*, and *Euschöngastia* mostly on small mammals. The grade of host specificity varies greatly by the species or groups, from those parasitic on various classes of vertebrates (such as some *Eutrombicula*) to those on only a single species of host.

Wharton (109), Lawrence (52), Wharton & Fuller (111), and Harrison & Audy (28, 29) emphasized that the apparent host-specificity of chiggers originated primarily from the habitat-specificity in that a species of vertebrate took the same ecological niche as a chigger species; for example, some *Euschöngastia* were known to breed in the nest or the passway of certain rodents. On the other hand, Sasa (83), Audy (5, 6, 7), Asanuma *et al.* (3), and others recognized that there is also a true or physiological host-specificity between some groups of vertebrates and chiggers. There have been a good many field observations of various authors to support this view. One of the most conclusive results was obtained through field experiments on Hachijo Island reported by Sasa *et al.* (91, 92). Among the several species of chiggers that occurred in the summer season, *Trombicula* (*Eutrombicula*) *wichmanni* (Oudemans) was the only species found on mammals (including human), birds, and reptiles, whereas the other species (including *T. hasegewai* and *Neoschöngastia* spp.) were all exclusively parasitic on birds. The authors found that all of these species were sensitive to carbon dioxide and could be recovered directly from their habitats by applying the experimenters' breath as the chiggers were seen crawling actively on the rock surface. Species other than *T. wichmanni* were found to act indifferently to rats or to human fingers, even when they were forced to attach, and soon left the body. However, they were apparently "attracted" to birds [chicken or *Uroloncha striata* (Linnaeus)], crawled actively toward the host, and were later recovered as engorged larvae. *T. hasegawai* and *Neoschöngastia* spp. were thus shown to have a physiological affinity to birds and not to other classes of vertebrates. On the other hand, *Neoschöngastia americana solomonis* Wharton & Hardcastle and *Neoschöngastia carveri* Wharton & Hardcastle, both of which had been known to attach exclusively on *Monticola solitarius* (Linnaeus) in this island, were found to have their specific habitat on rocks near the beach, thus resulting in the ecological host-specificity to the single species of birds. However, these species were also found to attach and engorge on chicken or *U. striata* (but not on rats) if experimentally put in contact.

*Site for attachment.*—Most chiggers tend to attach on hosts in closely packed clusters and on a specific site of choice. All chigger workers have recognized that the inner epithelium of the ears of rodents is a common site

of most species, but some prefer the lateral edge of the ears, the anal area, or the eye margin. Certain species have been collected only from peculiar sites like the tail of insectivores (83), the intranasal cavities of rodents (6, 9, 18), or the axillae of lizards.

The same species of chigger may attach to different sites according to the hosts. Cockings (12) reported that *T. autumnalis* on field voles was mostly found in the ears, but the same species on guinea pigs predominantly attached on the genital area. Sasa (83) also reported that *T. scutellaris* preferred the ears on rats, mammae on female dogs, the anal area on birds, and almost everywhere on humans.

*Feeding times.*—The time spent by larval chiggers engorging on a host differs by species of chiggers as well as by host. Nagayo *et al.* (71) observed that *T. akamushi* required one to three days to engorge on field voles, and Philip *et al.* (77) reported that *T. akamushi* and *T. deliensis* required some 40 hours, André (1) that *T. autumnalis* required 70 hours, and Wharton (109) that *E. indica* required 10 to 32 days to engorge on rats. Melvin *et al.* (63) observed that *T. alfreddugèsi* engorge on chicks, on the average, in 5.9 days, while on horned lizards the average time was as long as 30 days. Tanaka *et al.* (100) reported that the time required by *T. scutellaris* to engorge was longer and the recovery rate of engorged larvae was higher when attached to *Microtus montebelli* than to the other three species of rodents, mice, rats, and *Meriones unguiculatus* Milne-Edwards. Sasa *et al.* (91, 92) noted the difference in the feeding times by observing that *T. scutellaris*, *T. akamushi*, and *T. wichmanni* engorged in about two days on rats and man, whereas *T. hasegawai* and *Neoschöngastia* spp. required about a week to engorge on birds.

A comprehensive study on the feeding times was made by Harrison (26, 27) with some 40 species of Malayan chiggers. He developed a method of estimating the mean, maximum, and modal times for engorgement of chiggers from three different sources: laboratory-bred chiggers fed on laboratory animals; those found on marked rats that had been trapped within two days of release; and those on trapped wild hosts of unknown origin. There methods were applied to estimate the chigger population and also to demonstrate the difference in feeding times by the chigger and host species.

## Epidemiology

*Chiggers that attack man.*—Certain, but not all, species of chiggers habitually bite humans and may cause dermatitis or trombidiasis. Wharton & Fuller (111) listed 23 species of *Trombicula* (including subgenera *Eutrombicula*, *Blankaartia*, *Neotrombicula*, *Leptotrombidium*, and *Trombicula*), two species of *Euschöngastia*, three species of *Schöngastia*, two species of *Acomatacarus*, and one species of *Apolonia* as having been reported to attach on man. Several additional species were later reported to attack humans, such as *T. scutellaris* [Asanuma (2); Sasa and co-workers (83, 94)] and *E. xerothermobia* [Kepka (46)].

Chiggers are but the accidental parasites of man. The species that habitually attack humans are usually the group that takes a wide range of hosts, covering both mammals and birds or even reptiles. The skin reactions caused at the site of the bite may differ greatly by the species of chiggers. Large erythema, or even vesicles in some sensitive individuals, are known to be caused by species of *Trombicula* (*Eutrombicula*), *Schöngastia*, and *Acomatacatus*. Members of *Trombicula* (*Leptotrombidium*), including the vectors of scrub typhus, were reported by the classical Japanese workers, as well as by Fuller (22) and Philip *et al.* (77), to cause only a slight skin reaction. Sasa (83) recognized the difference between the reactions caused by *T.* (*L.*) *akamushi* and *T.* (*L.*) *scutellaris* by his own experience. The former was slightly painful but not itchy, whereas the latter was slightly itchy but not painful; both caused tiny erythema of a few mm. in diameter.

*Chiggers that transmit scrub typhus.*—Among the some 1000 species of trombiculid mites, those known to transmit the causative rickettsia of scrub typhus to man under natural conditions are confined to only a few species in the subgenus *Leptotrombidium*. K. Tanaka (102) was the first worker who unequivocally proved that the disease was actually transmitted by the bite of the mite and that the species that attacked man was not the same as those found on field vole in the endemic foci. Nagayo *et al.* (71), Kawamura (42), and Philip (75) reported that among the several species of chiggers recovered from field voles, only *T. akamushi* acted as the vector to man, though the others might be involved in the transmission among reservoirs. As was reviewed by Philip (76), Wharton & Fuller (111), and Audy (4, 7), results of the extensive survey made by various workers in South Asia and the Pacific regions also agreed in that the major vectors of scrub typhus were represented by *T. akamushi*, *T. deliensis*, and their closely allied forms, which constituted a well-knit group closely related morphologically.

The information pertaining to the occurrence of scrub typhus transmitted by species other than the *akamushi-deliensis* complex has recently been accumulated in Japan. Kuwata *et al.* (51) reported on the outbreak of the disease in the autumn among troops maneuvering at the foot of Mount Fuji, an area that was free from *T. akamushi*. Later an actue exanthematic disease prevalent among inhibitants of the Izu-shichito Island south of Tokyo in the autumn-winter season was shown to be a new type of scrub typhus by members of the Shichito Fever Committee (94). Sasa (81, 82, 83) and Asanuma *et al.* (3) accumulated epidemiological and experimental evidence that *T.* (*L.*) *scutellaris* was the sole vector of this type of scrub typhus, which was also found to be endemic in other areas of Japan, including the Fuji area. Fukuzumi & Obata (21) and Obata *et al.* (72) reported a case of scrub typhus from Kanagawa and incriminated *T.* (*L.*) *pallida* as its vector. Another type of scrub typhus was discovered in the coastal area of Shikoku Island by Sasa & Kawashima (85) and later by Hamada (25); this type occurred mainly in the summer season and was associated with high fatality.

Epidemiological evidence accumulated by Sasa & Kawashima (85), Kunitomo (50), and Yamaguchi *et al.* (114) support the view that *Trombicula* (*L.*) *tosa* Sasa & Kawashima is the vector, since it represents the sole species restricted to the endemic foci in this season of the year. Sasa (82, 83) reviewed the information assembled by these workers and concluded that there are at least several types of scrub typhus, all characterized by the epidemiological pattern being closely associated with the ecology of each vector species.

Based on evidence accumulated by various authors, chiggers may be grouped into several categories by the role played in the transmission of scrub typhus. Sasa (82, 83) and Audy (7) drew a similar interpretation working on different materials: (*a*) The efficient vectors, such as *T. akamushi*, *T. deliensis*, and *T. scutellaris*, are representative of the group that habitually bites man and is physiologically capable of transmitting rickettsia. (*b*). Some mammal-chiggers (especially the other members of *Leptotrombidium*) may be responsible for enzootic infection but do not, or only accidentally, transmit it to man, because they do not, or only rarely, attack man; this may be attributed to their host-specificity, their ecological niches being outside of human habitats, or their low populations. (*c*) Certain chigger groups are physiologically not capable of transmitting the rickettsia, even though they may be efficient human biters; such are probably represented by the species of *Eutrombicula* and *Schöngastia*. *T.* (*E.*) *wichmanni*, for example, is the common scrub-itch mite in the south Pacific, which is infested by scrub typhus; McCulloch (61) in Queensland reported that this mite was common in those areas that were free from scrub typhus and was absent from the endemic foci; Sasa *et al.* (81, 82) in Hachijo Island, Japan, reported that it attacked man only in the summer, while scrub typhus in this area occurred exclusively in the winter when *T.* (*L.*) *scutellaris* was prevalent. Philip (76) fed the closely related *T.* (*E.*) *splendens* on infected rats but failed to recover the rickettsia from the next generation. No scrub typhus has ever been reported in regions other than South Asia and Pacific, though scrub-itch mites of *Eutrombicula*, *Neotrombicula*, or *Schöngastia* may be common all over the world.

*Endemic regions and foci of scrub typhus.*—The distribution of scrub typhus in space and time is solely limited by that of the vectors, and its epidemiological pattern is a beautiful reflection of the ecology of mites, especially of the unengorged larvae. As was reviewed by Philip (76) and Audy (7), geographical distribution of scrub typhus is confined to the roughly triangular area connecting Japan, west India, and Australia. Although the basic observations on the epidemiology of the disease in each endemic foci were made by the prewar and the wartime investigators, more comprehensive studies into its nature were recently compiled by Audy and co-workers in Malaya and by Sasa and co-workers in Japan.

Audy (4, 7) and Audy & Harrison (8) classified the nature of scrub typhus by its origin into the following two types: the endemic scrub typhus, which

is largely a man-made disease associated with dense colonies of field-dwelling rodents, and the two major vectors, developed as the result of extensive deforestation; and the jungle tsutsugamushi disease, which is more primitive and complex, normally present as a silent enzootic cycle involving other chiggers and hosts. Audy (6) further proposed the explicable hypothesis that scrub typhus had evolved with the genus *Rattus* and the chigger subgenus *Leptotrombidium* and that the endemic area was limited as the distribution of the major vectors was barred by ocean, desert, or cold climate.

The endemic areas and foci in Japan are, as was reviewed by Sasa (82, 83), more complicated than in the tropics, since they involve at least several species of vectors of remarkably different ecology. The classical or Niigata-type of the disease transmitted by *T. akamushi* is confined to the alluvial banks on certain rivers in northwestern Honshu, which are covered by grasses and inhabited by dense populations of the field vole, *Microtus montebelli*. The Shichito-type transmitted by *T. scutellaris* is disseminated in southern Japan on various types of land, such as woods, cultivated fields, and sometimes even residential areas. The third, or Shikoku-type, occurs associated with *T. tosa* and is restricted to some coastal zones in Shikoku. The other types, including the Tsurumi-type probably transmitted by *T. pallida*, may occur widespread in association with the occurrence of the members of *Leptotrombidium* and are largely enzootic and more or less accidental in causing infection to man.

*Seasonal incidence.*—The incidence of scrub typhus is highly correlated to the population of vectors in the endemic foci, and thus it usually shows marked seasonal fluctuations. Among various climatic changes associated with the seasons of the year, temperature seems to act as the main limiting factor in the temperate zones, whereas precipitation is apparently more important in the tropics.

In the early stage of tsutsugamushi disease research in northwestern Japan, Nagayo *et al.* (71), in Yamagata, and Kawamura (42), in Niigata, already illustrated that the disease occurred exclusively in the summer season (from late June to September) when *T. akamushi* was abundant on field voles. Recently, Teramura (104) reported the seasonal distribution of the disease and the mites in the northernmost endemic foci in Akita. Morishita (67, 68) reported the monthly incidence of the disease in the Pescadores and in Formosa. Sasa (82, 83) compiled the monthly case records of the past 30 years of the three prefectures and made a comparative study with the reports by Morishita on the subtropical regions; Sasa concluded that the scrub typhus transmitted by *T. akamushi* was the summer type in its nature, its occurrence being limited to a very short summer period in northern areas, while it appeared earlier, lasted longer, and disappeared later as its locality moved southward, until it occurred throughout the year in the southernmost regions of Formosa.

The seasonal incidence of the Shichito-type scrub typhus, as well as that of *T. scutellaris* reviewed by Sasa (81, 82, 83), is interesting in that it

is the autumn type in its nature; the disease and the mites in northern localities, such as in the endemic foci near Mount Fuji, have occurred during short periods from early November to late October; in southern localities, such as in Hachijo Island, they appear much later, usually in late November, but their occurrence is for a longer period, until both the mite and the disease disappear in March or April. In other words, the disease and the mite first appear in the season when the average temperature drops to a level of about 20°C. and again disappear as it further drops to about 10°C., thus resembling the seasonal activity of some crickets or the flowering of chrysanthemums.

The seasonal incidence of scrub typhus in the tropics was reviewed by Audy (7), who concluded that precipitation or soil humidity was the main limiting factor. In the Indo-Burma region, there is a distinct typhus season in a monsoon climate from July to October; in the Malaya-Sumatra region, transmission takes place throughout the year, though minor fluctuations attributable to drier or wetter periods may be observed.

*Age and sex distribution.*—An interesting feature in the epidemiology of scrub typhus in relation to the local distribution of the vectors was pointed out by Sasa (82, 83), who compiled the age and sex distribution of the reported cases from Japan and from Formosa. The first type is that observed in the three prefectures in northwestern Honshu and on the mainland of Formosa, where the endemic foci infested by the mite-vector (all represented by *T. akamushi*) are confined to wild lands outside of residential areas and the incidence is highest among young adults and higher in males than in the females. The second type is that represented by Hachijo Island (autumn type transmitted by *T. scutellaris*) and by the Pescadores (summer type transmitted by *T. akamushi*), where the vectors are distributed all over the land, including the residential areas, and the incidence is highest in infancy with no significant difference by the sexes. In contrast to the seasonal incidence, which is attributable to the species-specific characters of the vectors, the topographical distribution of the vectors which affects such remarkable differences in age and sex distributions is apparently independent of the species of the vectors but is attributable to the local environmental factors.

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# THE PHENOMENON OF INDUSTRIAL MELANISM IN LEPIDOPTERA[1]

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Amongst all living things it has fallen to the Lepidoptera to provide the most striking evidence of rapid evolutionary change, a change that man has been able to witness in his lifetime. Yet the story of industrial melanism is one of lost opportunities. The reason for this is evident: it lies on the borderland of the entomologist, the collector of aberrations, the geneticist, and the field ecologist; it is in fact necessary to have an extensive knowledge of the Lepidoptera as well as scientific training. Furthermore, it frequently demands study in the open for 24 hours a day. It is possible that in the next 100 years the Lepidoptera will provide material in the field for the students of evolution and polymorphism of the same magnitude as *Drosophila* has for the cytogeneticists in the laboratory.

## Definition

Melanism in the Lepidoptera must refer to any instance in which there is an increased deposition of dark pigmentation in the wings or other structures of the insect. Usually melanism is controlled genetically; more rarely it can be, in certain species, brought about by the direct effect of environment on the insects. This latter situation plays no part whatsoever in industrial melanism, which is brought about by the indirect effects of industrialization through natural selection. Attempts have been made in the past to subdivide melanic Lepidoptera into those that were "true melanic," "melanistic," or "melanachroic" [Harrison (25)], according to whether they were all-black, blackish, or dark whilst maintaining their normal pattern. Examples of all these are found amongst the "industrial melanics"—moths that are changing their light-coloured patterns to dark ones because of the effects of industrialisation.

## Classification of Melanism

Melanic Lepidoptera fall fairly clearly into three headings: (*a*) industrial melanics; (*b*) relict or geographic melanics; and (*c*) semilethal melanics [Kettlewell (41)].

*Industrial melanics.*—These have manifested themselves only in the last 120 years and have been recorded nearly always in the first instance in or near industrial areas. They are characterised by rapid spread, by increased vigour, and, in the majority of species, by having no intermediate forms.

[1] The survey of the literature pertaining to this review was concluded in March, 1960.

They occur only amongst those Lepidoptera that depend on crypsis for their survival and that spend the hours of daylight sitting motionless on backgrounds such as lichened trunks, rocks, or dead wood. Their inheritance (except in a few instances) is one of simple Mendelian dominants. Industrial melanism has rightly been referred to as a transient polymorphism [Ford (12)].

*Relict or geographic melanics.*—In this group the melanics occur in a state of balanced polymorphism within their populations, as they have in all probability done, but at varying frequencies, for many thousands of years. They include those species that are found in normal but specialised parts of the world, such as on mountain tops or at high latitudes. In the few instances known, these dark forms are also inherited as Mendelian dominants.

It is hoped that the present review will provide evidence that the origin of our modern industrial melanics has links with these relict melanics of the past.

*Semilethal melanics.*—These occur as rarities, probably at about mutation-rate in certain species. The method of inheritance is recessive. They are usually subviable. They are found for the most part in those species that have methods of survival other than crypsis, for example warning colouration (Arctiidae and Zygaenidae). They may be as black as the melanics in other groups.

In assessing the position of industrial melanism, it is important to realise, therefore, that it falls into pattern with other types of melanism found in the Lepidoptera, but these other types have no connection whatever with industrialisation. Thus blackness can confer advantages under a variety of different conditions, for example in the high rain forests of South Island, New Zealand, where the rainfall may be 340 inches per year and the humidity high; or on cold mountain summits where "black" absorbs more heat, which increases the basal metabolic rate and permits more activity; or again at high latitudes during the summer months where, because of lack of darkness, all moths have to fly in daylight. We have evidence that heavy predation actually takes place by gulls whilst moths are on the wing during twilight. Finally, it has been observed that dark-coloured moths are less conspicuous in flight under certain conditions and this has been referred to as "aerial crypsis" [Kettlewell (43)].

It is indeed unfortunate that industrial melanism has been regarded previously as a unique phenomenon governed by laws of its own and dissociated from other kinds of melanism occurring in nature.

## Previous Work on Industrial Melanism

Toward the end of the last century, the effect of industrial melanism made itself felt and was mentioned in many entomological journals in Britain. It must be remembered that at that time Mendel's Laws of particulate inheritance (49), though published, had been overlooked and were in fact unknown until 1900. It is not surprising then that in attempting to explain the

origin of industrial melanism the emphasis was placed on environmental causes. Toward the end of the last century, Chapman (6), Cooke (9), and others vied with each other in suggesting different explanations, each with a Lamarckian bias. Tutt (61), on the other hand, preferred an explanation on the basis of natural selection. The issue was further complicated by a failure to appreciate the differing requirements of day-flying butterflies and night-flying moths. Hence the temperature experiments on the former by Merrifield (51, 52), Weismann (63), and others, in which melanic butterflies appeared, were quoted as proof of the direct effects of temperature and therefore applicable to industrial melanism in general. More usually the humidity factor was stressed.

In 1900, the Evolution Committee of the Royal Society commenced an investigation into the problem of industrial melanism. It is unfortunate that, in subsequent publications, actual figures were given only on two occasions, and that percentages or pious comments on the matter were the general rule [Doncaster (10)]. The same unfortunate shortcomings are found in the investigations of Mera (50) and of Adkin (1) in 1925. In fact, the frequency totals of samples, which would have helped so much at a later date, were never given. Nevertheless, industrial melanism was destined to come to the fore of biological science in the next few years in a way that was to attract considerable attention.

In 1926, Professor Heslop Harrison (23, 24, 26, 27) reported that he had fed stock of *Selenia bilunaria* (Esper) and *Ectropis bistortata* (Goeze) on foliage impregnated with lead nitrate and manganese sulphate and that he had produced a number of melanic offspring. He attributed this to the direct effects of the salts on the "soma," which in turn changed the "germplasm." This was pure Buffonism (incorrectly called Lamarckism) and came at a time when the biological world was as yet noncommital on the possibility of the inheritance of acquired characters; hence, the importance of Harrison's experiments. As evidence of an increased (and induced) mutation rate, Harrison stated that his melanics had occurred in a non-Mendelian ratio; in fact, there were far too few. He also reported that they were less viable than f. *typica*. Ford (13) was quick to point out that the melanics were inherited as Mendelian recessives and that, in view of the admitted subviability, the deficiency was to be expected. Fisher (11) also observed that if one had to accept Harrison's work as evidence of an increased mutation, it would demand a rate of 8 per cent, whereas the highest known rate in natural populations was one in 50,000 [Haldane (19)].

Hughes (32) repeated Harrison's experiments in 1932 and bred 3265 individuals under conditions identical to those of Harrison; Hughes concluded that "no melanic individuals have appeared either in the treated or the controlled broods." Thomsen & Lemche (60) also repeated Harrison's experiments on a large scale and stated: "The question which we originally posed, whether the methods used by Harrison could be explained as mutations with any degree of certainty, must therefore be answered in the nega-

tive" (translation). Others have repeated his work but on no occasion with positive results. Harrison's interpretation can therefore not be accepted. Furthermore, both his melanic forms were inherited as recessive characters and therefore bore no relationship to the industrial melanic situation in which this type of inheritance is extremely rare. More recently, Harrison (28) has referred to a specific "melanogen" as being the cause of industrial melanism, but he has been unable to substantiate this. Induced melanism must therefore be discounted as being responsible either for the cause or the spread of industrial melanism.

In Germany about the same time, Professor Hasebroëk (29) was conducting experiments on a rather similar line. He used the gaseous fraction, however, and not the solid portion of air pollution. Thus, the pupae of eight Rhopalocera and four Heterocera were subjected to the gasses hydrogen sulphide, ammonia, and pyredin in varying proportions. He claimed that melanism was produced in treated stock (and he produced photographs of these) but not among the controls. In no instance does an insect resembling a melanic appear, nor could any of his material (with the exception of two species) have any connection with industrial melanism. He failed to recognise the natural history of the phenomenon and hence primarily used butterflies as the subjects of his experiments. Furthermore, there is no evidence given as to the subsequent inheritance of his so-called melanic forms. He concluded that melanism was caused by the inhalation of various gases during respiration. While admitting the possibility that the conditions imposed by his experiments could have directly affected the pigment chemistry of individual pupae, Hasebroëk's conclusions on the origin of melanism can have no foundation in fact. Therefore, the claims of Harrison and Hasebroëk—that the effects of industrial environmental conditions were directly responsible for industrial melanism—cannot be accepted.

In 1943, a series of controlled experiments showed that the darkest forms of *Heliothis peltigera* (Schiffermueller) were produced by subjecting the pupae to the minimum temperature compatible with life for as long a period as possible, thereby lengthening imaginal development. Conversely, the whitest specimens of *H. peltigera* were brought about by heat and a rapid metamorphosis [Kettlewell (34)]. This had no bearing on industrial melanism, however, though it did confirm that, under certain conditions, environment alone could produce dark individuals in only certain species.

From 1930 onward, more and more melanic forms of Lepidoptera were recorded, but little was written on the theory and origin of the industrial melanics. In 1937, Ford (13) put forward, for the cause of industrial melanism, suggestions that for the first time conformed with known laws that were applicable to the evolution of other living creatures. These suggestions depended on two main evolutionary mechanisms: firstly, a constantly recurring mutation rate; and, secondly, natural selection, which decided the destination of the new mutants. Ford argued that black individuals did not find themselves at a cryptic disadvantage on modern industrial backgrounds, but

that industrial melanics in general had a superior viability. They were hardier, hence their advantage. This was a great step forward; no longer did new laws have to be invoked to account for the phenomenon of industrial melanism. Ford (14) and Onslow (55, 56) were the first to show that industrial melanics had, apart from the colour change, a different physiology leading to an increased viability. Ford worked on *Alcis repandata* (Linnaeus) and Onslow on *Ectropis consonaria* (Hübner), *Boarmia punctinalis* (Scopoli), and *Deileptenia ribeata* (Clerck). All were true industrial melanic species. This was indeed a great advance, but it left many aspects unexplained and unproven. What was the cryptic advantage or disadvantage of the industrial melanics from the point of view of predators? Why the rapid spread, and why was the melanic character nearly always dominant?

## Distribution of Industrial Melanism

At the present time, industrial melanism is found in many entirely disconnected places in the world, and, in each instance, it occurs in cryptic species only. In Britain, which was the first country to record it, it occurs today not only in all industrial areas, but far beyond. But in Britain this melanic spread is always toward the east. In *Biston* (=*Amphidasis*) *betularia* (Linnaeus), the peppered moth, it has been shown that the cline to the west is sharp, but to the east it is long and gradual. Evidence has been provided that this distribution pattern is an indirect result of the prevailing southwesterly wind, which day and night carries for great distances the lighter portions of air pollution—smoke—from industrial areas toward the east [Kettlewell (35)]. Similar situations exist in and around most industrial areas throughout the northern hemisphere. In Europe, industrial melanism was recorded in the Rhine Valley about the same time as in Britain, and a history of its spread can be obtained from Gerschler (16), Hasebroëk (30), Walther (62), and Heydemann (30a). Industrial melanism is widespread throughout the North American continent. In the Pittsburgh area alone, it is probable that more than 100 species of Lepidoptera are in the process of becoming melanic. In Canada, industrial melanism is found around Montreal, Toronto, and probably elsewhere [Kettlewell (43)]. In the highly industrialised districts of eastern Europe, in Yugoslavia, Czechoslovakia, and Poland, industrial melanism is again widespread. In fact, it probably occurs in all industrial regions of the earth except the tropics, and it is of interest that, so far, no records have come from there. It may be that the advantages of camouflage are outweighed by the disadvantages of excess black pigment under conditions of great heat.

## History of Spread

Black specimens occurring in previously light-coloured species were first noted about the middle of the last century. One of the first and best documented of these was the all-black form of the peppered moth, f. *carbonaria* Jordan (=*doubledayaria* Millière), which occurred in 1848 in Manchester.

This species was at that time considered an insect of considerable rarity. By 1898, fifty years later, we have evidence that this same black form occupied about 95 per cent of the Manchester population. Haldane (18), assuming that in 1848 the melanic frequency could not have been higher than 1 per cent, showed that this reflected an approximate 30 per cent advantage of the black form over the light for this period of time in Manchester. A selective advantage of this magnitude had not previously been known to occur in the wild. The form *carbonaria*, which is inherited as a Mendelian dominant, spread rapidly through central industrial England but nearly always in the rear of a second, but less extreme melanic, f. *insularia* Thierry-Mieg, another dominant to which f. *carbonaria* is epistatic. It has recently been shown that these two forms are not allelomorphs [Kettlewell (37)]. In the London area, f. *carbonaria* did not appear until 1897, and once again spread with great rapidity. Fuller accounts of the spread are given by Adkin (1), Barrett (3), Doncaster (10), and Harrison (22).

I have recently completed a survey of the frequencies of *B. betularia* and its two melanic forms throughout Britain [Kettlewell (43)], and the frequency map, constructed from over 20,000 records, shows that only in the west of England and north Scotland is f. *typica* still found as 100 per cent of the population. The map provides evidence that there is a correlation between industrial areas and a high proportion of melanics. It also demonstrates conclusively that throughout the eastern counties, though far removed from the industrial centres of Britain, the *carbonaria* frequency never drops below 80 per cent of the population. It was our earlier lack of understanding of this reflected long-distance pollution drift that led many entomologists to doubt if so-called "industrial melanism" really had any connection with industrialisation.

It may be added that in North America the cospecies of *Biston* (= *Amphidosis*) *cognataria* (Guenée), with its melanic form *swettaria* Barnes & McDunnough, has more recently behaved similarly.

In Britain there are approximately 780 different species of macrolepidoptera, and, of these, between 70 and 100 are undergoing the same process of substituting their highly complicated and specialised patterns, built up to perfection over thousands of years, for dark or black colouration. New melanic forms are being recorded each year, and we are able to watch a steady increase in the frequency in some of these. On the grounds of theory, I have prepared a graph showing the expected rate of spread, given certain conditions (Figure 1). The following observations can be made: Firstly, the period from the original mutation until the new mutant reaches 1 per cent of the population is a long one; during this time the gene-complex will be adjusting itself to the new mutant. Secondly, having reached the level of 1 per cent, the mutant thereafter will spread through the population at a great speed. Thirdly, when the mutant reaches 80 to 95 per cent the rate of spread will decrease or possibly cease altogether, depending on whether the heterozy-

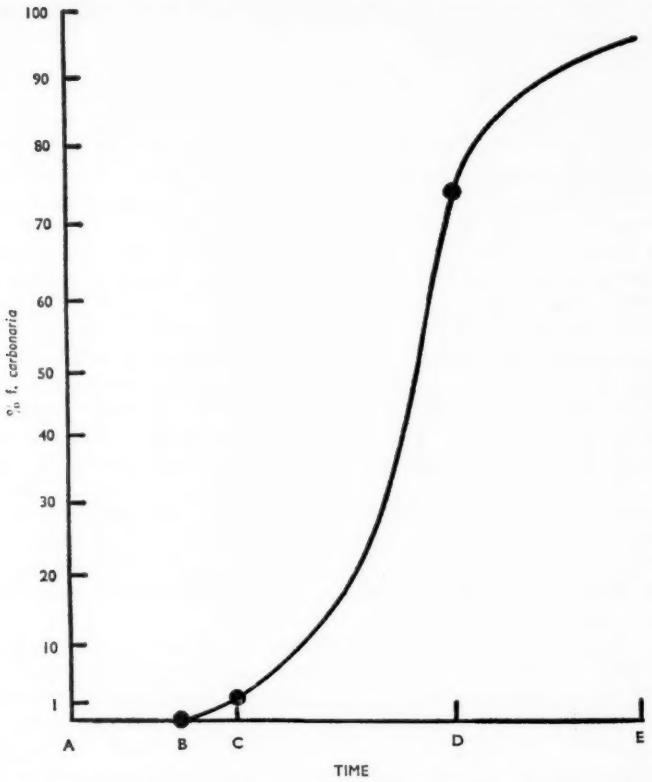


FIG. 1. The rate of increase of a melanic (dominant) mutant with a mutation rate of one in a million and with a 30 per cent selective advantage over its typical form. A constant advantage of the heterozygote is assumed throughout, although in practice this will not occur.

gotes are physiologically better suited than either of the homozygotes. Now the observed facts to date suggest that the rate of spread does in fact follow the path of this sygmoid type of curve. Collectors have repeatedly recorded initial melanic captures as rarities for a considerable period of time, and they are repeatedly subsequently astonished at the suddenness of spread. Lastly, in regard to *B. betularia*, nowhere has f. *typica* completely disappeared, not even in Manchester, where f. *carbonaria* has for more than 60 years occupied 95 to 99 per cent of the population. Slow initial inception, followed by a rapid penetration, and finally slow elimination (or a balanced polymorphism)

would appear to be the process, certainly in regard to *B. betularia* and its melanic form *carbonaria*.

## Air Pollution

The spread of industrial melanism is the indirect consequence of pollution fallout. For the first time in history, widespread man-made ecological changes have taken place insidiously, following the Industrial Revolution in the latter half of the eighteenth century. Nevertheless, these changes have been so rapid that in some species they have caused local extinction.

It is well then that we consider air pollution itself, firstly at its origin, secondly at its ultimate destination, and thirdly its direct effects. The Department of Scientific and Industrial Research (2), who are responsible for measuring the amount of air pollution in Britain, arbitrarily divide the fallout into "heavy" and "smoke." The former is measured in tons per square mile per month in and around its origin, and many industrial centres receive up to 50 tons per square mile per month. The latter portion, "smoke," is measured in milligrammes per 100 cubic metres of air, which is sucked through a filter. It is the insidious effects of the second portion—drift smoke —that have largely been overlooked, firstly in regard to the biological significance and secondly, until recently, in regard to its long-distance effects. There is evidence, in fact, that "smoke" travels very great distances and that after day and night fallout for a hundred years or more its effects are profound. The idea of widespread dissemination of small particles is endorsed by a recent accidental experiment.

On October 10, 1957, the Windscale reactor incident took place: radioactive particles escaped into the atmosphere near ground level [Murgatroyd (53)]. This is probably the first unplanned marker experiment in which radioactive elements were released into the air in a way other than by atomic explosion. The ultimate destination of the particles then bears some relationship to the distribution of the lighter portion of air pollution and the way in which it is carried. Subsequent analyses showed that the radioactive count was increased (though not to danger limits) as far away as Scandinavia, France, northern Italy, and West Germany. This then is an indication of the distance that small particles can travel. It is likely, with the prevailing southwesterly wind in Britain, that the majority of our smoke falls into the North Sea. It is not surprising then that we have found evidence of pollution effects throughout the eastern counties of Britain, though they are far removed from the centres of origin.

## Effects of Air Pollution

The smoke particles get caught up on the leaves and twigs of trees and bushes. They are also precipitated on the ground. The amount is affected by many variables—the degree of pollution fallout, rainfall, and the number of aphids present on the leaves. It follows that evergreens will become dirtier

than deciduous trees because they receive both winter and summer precipitation.

The canopy of all trees is washed by rain, and the pollution is carried down the boughs and finally the trunk. The first indications are seen on the underside of boughs, which show dark "rain runs." All vegetative lichens are rapidly killed and this probably happens at low dilutions. The trunks become bare and finally black. I have recently dug, to a depth of two inches, sample cores of the "earth" around the foot of trees in industrial Yorkshire. Up to a distance of two feet from the trunk certain samples consisted of nearly pure pollution. This accounts for the fact that no vegetation will grow around the foot of trees in industrial centres. Pollution drift also cuts down the amount of light, and this has a considerable effect around all manufacturing districts.

In the last few years we have attempted to analyse the direct effects of air pollution. By collecting leaf samples (six ounces) from the same tree at monthly intervals and washing them, we have found that in deciduous trees there is a relation between the amount of pollution present and the lateness of the season, i.e., the leaves are dirtier in September than in April. Other considerations must be taken into account. We have found that when a tree becomes infested with aphids, apart from sooty moulds which grow in their sticky secretion, pollution itself is held more readily. We have also been studying the direct effects of pollution on lichens. By using a transparent tape measure to encircle the trunks of random trees (only the same tree species can be compared) we have been able to undertake comparative lichen counts. A correlation between the total number of lichens present (and the number of species) and the distance from an industrial centre was first recorded by Jones (33). These investigations have led us to believe that air pollution is responsible for profound ecological changes. Apart from killing the vegetative lichens and blackening tree trunks and boughs, in some places, such as Sheffield, England, the ground itself, along with the fallen twigs and dead leaves, becomes darkened. All these are the normal resting places for the majority of cryptic moths. In 150 years, the backgrounds have entirely altered. At the same time, considerable selection against the toxic effects of heavily polluted foliage, which the majority of larvae have to eat, must be taking place. This, then, is the background of the changed environment in which industrial melanism has arisen.

## Genetics

Probably over 90 per cent of industrial melanics are controlled by a single gene, and the melanic character is inherited as a Mendelian dominant; but rarely today do intermediate forms occur. The genetics of many industrial melanics have been determined: for example, *Gonodontis bidentata* (Clerck) f. *nigra* Prout by Bowater (4), *Hemerophila abruptaria* (Thunberg) f. *fuscata* Tutt by Onslow (57), and *Alcis rhomboidaria* (Schiffermueller) (= *gemmaria* Brahm) f. *rebeli* Aigner by Williams (64); all have complete dominance.

Other mechanisms, however, have been found to promote darkness. Multifactorial inheritance is found in the moth *Lymantria monacha* Linnaeus, the darkest forms of which are controlled by three genes, two autosomal and one sex-linked. All are dominant, and each confers a degree of darkness. These genes do not occur simultaneously in Britain, though they do in Germany [Goldschmidt (17)]. The geometrid moth *Oporinia dilutata* (Schiffermueller) produces a series of varying melanics, and it is stated that these are controlled multifactorially [Harrison (22)]. Multiple allelomorphs are responsible for the melanic forms of the European moth *Aglia tau* (Linnaeus) [Standfuss (59)] as well as the tortricid *Acleris* (=*Acalla*) *comariana* (Zeller) [Fryer (15)].

Female inheritance is found in the geometrid moth *Phigalia pedaria* (Fabricus) [Bretschneider (5)]. In this species the female is apterous, and one type of inheritance is the normal Mendelian, in which both sexes can be black. There is another, however, in which black female *P. pedaria* beget melanic females only, the males remaining f. *typica*. This character could therefore be carried either in the Y chromosome or in the cytoplasm; at present we do not know which.

Polygenic inheritance has been overlooked in the past. Nevertheless, there is circumstantial evidence that it takes place. The geometrid moth *Gonodontis bidentata* has a dominant black form *nigra* [Bowater (4)]. This species does not fly very actively and the genes therefore spread slowly. At Cannoch, outside the city of Birmingham, over 50 per cent are of this melanic form. In the centre of Birmingham, however, this melanic mutant does not occur. It is probable that the primary mutation has not taken place. Form *typica* is found here, but is very much greyer and darker than that found in the rural *G. bidentata*, and there is considerable variation among the individuals themselves [Kettlewell (46)]. The same situation is found in a large number of noctuid moths, which have gradually been getting darker in the last 50 years. Polygenes and modifiers are constantly at work affecting the phenotypes of both melanic and typical individuals.

## The Build-Up of Dominance

Ford (12) has demonstrated that dominance is achieved by the selection of modifying genes. Furthermore, he showed that the modifiers that bring this about may differ if the major gene finds itself in similar but isolated environments. He used the noctuid moth *Triphaena comes* (Hübner), which has a very dark form *curtisii* Newman, with near, but not complete, dominance. Ford used stock from the islands of Balla and Orkney off the west coast of Scotland. By carrying out reciprocal crosses, in which he put the *curtisii* gene from one island into the gene-complex of the other, he produced broods in which there was no segregation but in which individuals varied from f. *typica* to black. This then demonstrated the breakdown of dominance—dominance which had been achieved in the wild by the natural selection of modifying genes. Now *T. comes* f. *curtisii*, though melanic, is not of industrial origin;

nevertheless, it presents an object lesson on how dominant melanics can be built-up. We have in fact other clear-cut evidence that this can take place rapidly in nature.

In the last few years, I have collected some of the earliest caught examples of *B. betularia* f. *carbonaria;* all were taken during the latter half of the last century. They are characterised by having white markings on both fore and hind wings and are quite unlike modern heterozygote f. ***carbonaria***, which today but rarely show a trace, except on the costa of the hindwing, which is always hidden when at rest. These forms are therefore intermediate in appearance between f. *typica* and f. *carbonaria*. Modern heterozygotes from industrial centres are all-black except for four white specks situated on the head and at the base of the forewings. In the last few years even these specks have disappeared from a portion of *carbonaria* specimens from city centres, such as Birmingham and Chester; these insects are entirely black. Haldane (20) has recently pointed out that this must have been brought about by the selection of modifying genes. Present day heterozygote *carbonaria* are indistinguishable from homozygotes; dominance is complete. This must have been brought about by intense selective pressure by visual predators. In order to test this in the laboratory, I have recently attempted to break down this dominance in the same way that Ford succeeded in doing with f. *curtisii*. Using heterozygous f. *carbonaria* from Birmingham, for three generations the melanics have been outcrossed to f. *typica* stock from Cornwall (western Britain), which had no recent previous experience of the *carbonaria* gene. The evidence to date shows that a proportion of the heterozygote f. *carbonaria* had either increased white markings or a dusting of white scales. These individuals are in fact unlike the majority of industrial heterozygote f. *carbonaria* found today.

In the same way, British melanic f. *carbonaria* are being outcrossed into the North American peppered moth (*B. cognataria*) with similar results.

The breakdown and build-up of dominance can therefore be demonstrated in the laboratory by intensive artificial selection in a comparatively few generations. What has not been appreciated is that the selective pressure in nature is probably very much greater than anticipated and that this same mechanism of build-up of dominance can take place in the wild, though not as rapidly, nevertheless at a very fast rate. There are in fact very few examples of intermediate heterozygotes among the industrial melanics. One of the few species which fall into this category is *Polia nebulosa* (Hufnagel), which passes the day at rest on tree trunks. Mansbridge (48) recorded that two melanic forms occurred at the beginning of the century in a localised area of Cheshire; one was grey with light thoracic scapulae f. *robsoni* Collins, which was the commoner; and he showed it to be the heterozygote of a second and rarer form *thompsoni* Arkle, which was the homozygote melanic. Strangely, this insect was fringed with pure white scales. It is in fact likely that about the year 1908 both homozygotes were at a disadvantage; the one, f. *typica*, because of its all-light colouration, the other, f. *thompsoni*, because

of its silhouette in white. Mansbridge (47) recorded that from wild larvae, collected between 1905 and 1914, 972 imagines were reared of which 142 were melanic, but only three of these were f. *thompsoni* [Bowater (4)]. There were, therefore, 830 f. *typica*. It appears either that there was a gross deficiency of the homozygotes or, what is more likely, that the majority of the "intermediates" were another, but phenotypically similar, mutant. In 1956, I was sent a sample of about 50 larvae collected from this same Cheshire wood and others that were bred at the same time. All the imagines were dark grey without white scapulae; this is probably f. *plumbosa* [Mansbridge (48)]. Nor have f. *typica* or f. *thompsoni* been bred or found in the wild for many years. It is likely that the original mutant, which had incomplete dominance, has been replaced by an entirely different one which is similar to f. *robsoni* but without white scapulae. The majority of specimens found in Yorkshire and elsewhere in the industrial Midlands is now of a similar form.

Intermediate heterozygotes that are recognisably distinct are rare among the industrial melanics, and the story of *P. nebulosa* is an unusual one and probably reflects a selective disadvantage of the homozygote, f. *thompsoni*, because of its white silhouette. We have shown that more frequently the semidominant mutation has obtained complete dominance under industrial conditions within a few years. These melanics have kept their semidominant state before their recent mutation because of previous experience in polymorphisms under very different but advantageous conditions in the past. During these periods, dominance for the melanic character has been brought about by the effects of natural selection on the gene-complex. Are there any species, then, where melanic forms are spreading under the industrial conditions of today but have played but little part in polymorphisms in the past? Is there any evidence of present-day success of an industrial melanic recessive? Though the two are not synonymous, in theory the one might imply the other. There are in fact recessive melanics that are found in populations today at a considerably higher frequency than that expected by mutation only. The black form of *Lycia hirtaria* (Clerck) appears to be on the increase in the London area, and melanic forms of several species that copy dead leaves, such as occur in the genus *Ennomos* and *Selenia*, and in *Gastropacha quercifolia* (Linnaeus), are more often taken in the wild. A most interesting example of rapid spread of a recessive melanic character, but one which has probably contributed only to local polymorphisms in the past, is *Lasiocampa quercus* (Linnaeus) f. *olivacea* Tutt [Kettlewell (46)]. Of 493 moths caught in 1957 on the heavily polluted Yorkshire moors, 5 per cent were of this form, so that about one-third of the population must have been heterozygous for *olivacea*. The normal light-yellow female spends the day at rest low down in the heather and is on the wing at night only. The male flies freely by day. I was able to show that one of the factors contributing to the spread of this dark form was its cryptic advantage both for females at rest on the darkened heather and also for the males in flight. Many hundreds of the females were seen to be eaten by flocks of black-headed gulls (*Larus ridibundus* Linnaeus),

which have only recently been recorded as spending the summer in central England. But this is not all. Collinson (8) has noted not only an increase of frequency of the recessive form since 1936, but also the gradual darkening of all *L. quercus* occurring on these moors. The situation is further complicated by the fact that the majority, but not all, of melanic moths are bred from melanic larvae. The genes controlling larval and imaginal melanism must therefore be linked. Form *olivacea* (or a similar form), which has increased so rapidly on the Yorkshire moors, occurs in the industrial areas of Cheshire and Lancashire. Black larvae occur here also. However, f. *olivacea* is found in other habitats dissociated from the conditions of industrialisation. It is common in Caithness, Scotland, where black larvae have not been found [Cockayne (7)] and where presumably this mutation has not taken place. Similar situations occur locally in Holland and Denmark [Hoffmeyer (31)]. On the estuary of the river Haval near Brandenburg in East Prussia, the black mutant is reported to form 100 per cent of the population [Niepelt (54)] and 100 per cent of the caterpillars bred from this stock are of the black form, though this is doubted by Cockayne (7).

It would appear, then, that *L. quercus* f. *olivacea*, previously confined to local polymorphisms usually situated near the coast, is now finding itself at a considerable cryptic advantage in present-day industrial conditions. It will be of the greatest interest to see what the future of this recessive mutant will be.

## The Evidence for Selective Predation

Whatever other characters are involved in the one-gene substitution that is responsible for industrial melanism, it must be admitted that the colour change to blackness must have great survival significance. In fact, it alone might account for the whole phenomenon. It is a strange fact that, in spite of an abundance of both amateur and professional ornithologists and entomologists, no evidence has been forthcoming in the past of birds predating cryptic moths while sitting stationary at rest. Accordingly, in 1954 a pilot experiment was carried out at Madingley Research Station, Cambridge, England, and it was shown that a pair of great tits (*Parus major* Linnaeus), housed in a large outdoor aviary, took and ate *B. betularia* selectively in their order of conspicuousness [Kettlewell (37)]. Subsequently, large-scale releases of both forms of *B. betularia* were undertaken in the wild. Firstly, this was carried out in a completely unpolluted wood in which the trees were covered with lichens and where 100 per cent of the wild population of *betularia* were f. *typica*. Secondly, I chose a heavily polluted wood near Birmingham in the centre of industrial Britain where only 10 per cent were of this form [Kettlewell (36)]. The experiments fell under the following three headings: (*a*) human assessment of the colour efficiency of the two forms in regard to the backgrounds of the tree trunks on which they were released; (*b*) direct observation of their ultimate fate; (*c*) use of mark-release-recapture techniques to ascertain the relative survival rate in each of the contrasting localities.

"On the oaks in Birmingham the *carbonaria* are nearly always extremely well protected, but the reverse is shown for the typical; the scoring on one is inversely proportional to that of the other" [Kettlewell (37)]. This was the conclusion reached as the result of the release of 651 male and female *B. betularia* onto the tree trunks near Birmingham. The complete opposite was concluded in the unpolluted and heavily lichened woodlands similar to those which probably existed throughout Britain 200 years ago. Here f. *typica* on nearly every occasion scored the highest of the six marks awarded for efficient crypsis.

Direct observation of the released moths showed that bird recognition was very similar to that of man. In the lichened woodlands, N. Tinbergen, who was helping with the experiments, observed 164 f. *carbonaria* eaten to 26 f. *typica* eaten after each form had been released in equality. Predators were the spotted flycatcher, *Muscicapa striata* Linnaeus, the nuthatch, *Sitta europaea* Linnaeus, the yellow hammer, *Emberiza citrinella* Linnaeus, the European robin, *Erithacus rubecula* Linneaus, and the thrush, *Turdus ericetocum* Linnaeus. In the industrial woodland we recorded a complete reversal and noted that among other birds, redstarts, *Phoenicurus phoenicurus* Linnaeus, ate 43 f. *typica* to only 15 f. *carbonaria*. A film record of all these experiments was made.

Finally, an over-all selective advantage was assessed from the recapture results. In the industrial woodland, a total of 630 *B. betularia* were released, 137 f. *typica*, 447 f. *carbonaria*, and 46 f. *insularia*. Each was marked with a dot of quick-drying cellulose paint; the colour varied according to the day. In these polluted woods, 27.5 per cent of f. *carbonaria* were recaptured to only 13 per cent of f. *typica*. In the contrasting unpolluted woodland, such as is still found throughout southwestern England, 984 *B. betularia* were marked and released, 473 f. *carbonaria*, 496 f. *typica*, and 15 f. *insularia*. Of the f. *typica*, 12.5 per cent were recaptured but only 6.3 per cent of f. *carbonaria* [Kettlewell (40)].

The experiments show that there can be no doubt of the cryptic advantage of f. *typica* in unpolluted and lichened woodland nor of f. *carbonaria* in industrial countryside. This fact alone could be sufficient to account for the rapid spread of industrial melanism. On the other hand, it would be very surprising if this were so.

## Physiological and Behavioural Differences

*Viability differences.*—An increased viability of the melanic forms has been noted by many workers in different species (*vide ante*), but all were using material in which melanic forms had been present within their populations for a considerable period of time. We have evidence that in the early days of industrial melanism the reverse was the rule, i.e., the earliest recorded backcross broods in the literature of *B. betularia* (f. *typica* × f. *carbonaria*) produced a deficit of f. *carbonaria* [Kettlewell (42)]. Haldane (21) has analysed this and has pointed out that, at this time, the gene-complex had had no

recent experience of the gene-conferring melanism. The melanic gene found itself in a gene-complex that had been built-up for the cryptic requirements of *f. typica* and not of *f. carbonaria*. On theoretical grounds, it has been suggested that modifying genes, previously committed to complicated cryptic responsibilities, are now freed to confer optimum physiological advantages [Kettlewell (44)].

In a series of experiments using backcross broods in which the larvae of *B. betularia* had to feed on polluted leaves, a significant excess of melanic forms survived. This conforms with previous viability findings.

*Mating preferences.*—Sheppard (58) has shown that with typical *Panaxia dominula* (Linnaeus) and the dark form f. *medionigra* Cockayne unlike matings were more frequent than like matings. In a short series of assembling experiments, it has been shown that females of *B. betularia* f. *typica* and f. *carbonaria* behaved entirely differently in regard to their capacity for attracting f. *typica* males, and that this varied according to the temperature [Kettlewell (43)]. The female assembling-scent of *B. betularia* is probably represented by a complex of molecules. At times it is capable of attracting male *Xanthorhoë montanata* (Schiffermueller) [Kettlewell (36)] and at others *Xanthorhoë spadicearia* (Schiffermueller) as well as males of its own species [Kettlewell (39)]. Differing female behaviour under varying environmental conditions is, however, more likely to account for assembling preferences than chemical differences between the scent of f. *typica* and f. *carbonaria*.

*Larval behaviour differences.*—Evidence has been provided that the larvae of the two forms behave differently. Normally in Britain the larvae can be found in nature from June to October. It was shown that in certain backcross broods of industrial origin those larvae that fed rapidly and pupated first formed a significantly higher proportion of f. *typica*. Conversely, those larvae that grew slowly and were still feeding in late September and early October, subsequently produced a significantly higher proportion of f. *carbonaria*.

This might reflect the result of selection that favours quick feeding in f. *typica*, which are less able to face the heavier degrees of pollution which cover industrial trees later in the year. Alternatively, the melanic form, by slow feeding, may be able to get rid of toxic materials. Examined in this light, industrial melanism might be considered a means of extending the larval feeding time under adverse conditions. Evidence that speed of larval feeding can, in part, be under the control of genes is suggested by the fact that the North American peppered moth, *B. cognataria*, of Canadian origin but bred in Britain, fed up much more rapidly than British stock. The reason for this might be the rapid onset of Canadian winters, compared with those in Britain, so that late larvae are regularly killed off. There has been selection for rapid development.

*Background recognition.*—Too little is known about the situations in which Lepidoptera pass the day. Choice of site must indeed have an important bearing on survival, for even the commonest moths are but rarely found at rest in the wild. Selection of resting sites and orientation to them—having

regard to the effects of direct sun and rain, predation, and casual attacks by ants—must surely demand a complex choice. The question posed is whether moths take up a random position in their favourite resting places, or whether they are capable of recognising backgrounds according to their own colour differences. In regard to industrial melanism, this would demand that each morph assess its own colour value and match it accordingly. The importance of the answer to this question cannot be overstressed. If random choice is the rule, the elimination of, for instance, f. *typica* would take place very much more rapidly in a locality where lichens were scarce. On the other hand, if the selection of a resting site is not fortuitous, provided that both habitats are available, polymorphism could be the outcome.

In order to test background recognition, equal numbers of light and dark *B. betularia* were released into a large tub which presented equal surface areas of black and white. Each morning the insects were scored according to the position they had taken up. A significant proportion ($\chi^2_{(1)} = 10.9$) of both forms chose correct backgrounds [Kettlewell (38)].

It has been suggested that a single mechanism, one of contrast appreciation, could be responsible for non-random choice of background in both forms. The theory is that, if the segment of the eyes that receives light from the circumocular tufts conflicts with that of the segment that receives the background colour, a state of "contrast conflict" would arise. Field observations have been recorded as evidence of this [Kettlewell (45)].

It is likely that in the future many other physiological and behavioural differences will be recognised. In theory, these differences are more likely to be found in situations where each form has existed in its population for a long period of time; that is to say that within the present context one should look for these among relict melanics, for here, because of particular environmental conditions, each form has continued to occur in a state of balanced polymorphism, but at varying frequencies, for many hundreds, or probably thousands, of years. Unfortunately, we have as yet little knowledge of the physiological and behavioural differences of these relict melanics.

## A Theory of the Origin of Industrial Melanics

That melanic and normal forms should confer advantages under entirely different environmental conditions is not surprising if one considers the basic physical differences between black and white. A non-specialised black colouration may offer many advantages over a highly complicated light one in a variety of separate habitats. Melanism is, in fact, a basic and recurring necessity and one which may arise from time to time for very different requirements. Is there any evidence then that the industrial melanics of today have any connection with melanic polymorphisms of the past? The answer is almost certainly yes. Melanic moths are found today in ancient Caledonian pine forests. These relict tracts are probably similar to areas that extended over much of Britain 8000 to 10,000 years ago, before the succession of the deciduous trees. In at least one species, *A. repandata* f. *nigra*, the melanic

form occurs at a frequency of 5 to 10 per cent. This form is phenotypically similar to (though recognisably different from) the industrial melanic that has recently spread through industrial England. Evidence has been provided that the balanced polymorphism that exists in unpolluted Scotland is in part attributable to the "aerial crypsis" which the black form enjoys while on wing [Kettlewell (43)]. Breeding experiments are at present in progress to test whether the black forms are different expressions of the same gene in their respective gene-complexes. Each has been shown to be inherited as simple Mendelian dominants.

Other species found throughout Britain, such as *Allophyes oxyacanthae* (Linnaeus) and *Nonagria dissoluta* (Treitschke), have black forms that occur at a low frequency everywhere. In industrial districts, however, these same forms behave as industrial melanics and their frequencies are greatly increased.

It would appear that the conditions imposed by man over large areas of the world today present a further opportunity for the spread of melanism. Though these bear little relation to environments of the past, the basic need for melanism, with non-specialised pattern, has once again arisen. Though melanic forms are excluded, except by mutation, from large areas of the world, they still occur in polymorphisms under completely natural conditions today. It is in situations such as these that dominance has previously been achieved. Conversely, the recessive melanics of non-cryptic species have never in the past experienced such opportunities; hence their recessive state. The gene-complexes have never had to adjust, nor have they often participated in a polymorphism. The melanic mutation probably reflects some particular and recurring line of chemistry, some specific enzyme, a fundamental change. As to the production of the melanin in Lepidoptera, however, we are at present largely ignorant. On the other hand, it has now been shown that industrial melanism, its origin, and its spread can be accounted for by the natural laws of genetics and natural selection.

This review has been presented with the object of pointing out the many lines of research that urgently need to be undertaken in a much neglected branch of genetics, ecology, and entomology.

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# A REVIEW OF THE PHYLOGENY OF MITES[1,2]

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## Introduction

Phylogeny stems from somewhere prior to Aristotle's Lyceum and Charaka's writings in Sanskrit, when man first devised means of recognizing, classifying, and relating organisms. Phylogeny, at best, is controversial, subject to personal interpretation, and, in some cases, highly speculative. It is the purpose of this review, however, to rehearse the major aspects of the postulated phylogeny of the mites and attempt to correlate and interpret this information. The extent to which these goals may be achieved is modified, obviously, by time, space, available literature, and the limitations of the writer. Since acarology is in its relative infancy, there is a paucity of literature on the subject, but, as more emphasis is placed on the group and as the understanding of the morphology, physiology, embryology, and paleontology of mites unfolds, their phylogeny will be more thoroughly understood.

From a general point of view, Bigelow (7), Bader (3), and Mitchell (45) elucidated pitfalls to be avoided regarding phylogeny. Among other things, they proposed that those who deal with phylogeny and taxonomy be careful to dispose of erroneous concepts and that they be certain that the fossils are considered. For most mites, the latter evidences are difficult to obtain.

A cogent statement regarding acarine phylogeny is made by Hughes (41):

> In effect, we are here dealing with the end products of long evolutionary histories and, in the absence of direct evidence of fossil forms, and with a complete absence of embryological knowledge of the forms concerned, coupled with the fact that the mite fauna is very incompletely known, it is evident that phylogenetic trees are certain to be largely illusory.

## General Relationships

Snodgrass (63) and Petrunkevitch (58) postulated that ancestral arachnids arose in the Cambrian Period, and changed essentially to chelicerate forms with a ventral and posteriorly displaced mouth, which basically resembled a eurypterid. They assumed that the Arachnida split into a number of orders at an early geologic age and, although fundamentally similar, developed with trends toward shortening of the abdomen by loss of the posterior segments. Petrunkevitch (58) reasoned, on the basis of fossil evidence,

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that those orders were primitive that exhibited the 12 original abdominal segments or the least deviation from that number; those orders with fusion or loss of these segments were more advanced. He also related changes in the coxae, mouth parts, and digestive tract.

According to Snodgrass (64) and Petrunkevitch (58, 59), the Acarina demonstrate external and internal structural relationships with the Arachnida. These include the six pairs of appendages (chelicerae, palps, legs.) Vitzthum (68, 69) assumed an arachnid origin for the mites and believed that the Mesostigmata are related to the Ricinulei or Podogona because of the six-legged embryo and the prosomal stigmata above coxae III in the Ricinulei.

Thor (65) considered it impossible for the Acarina to have been derived as a separate order of the Arachnida and assumed a distinct origin and divergent development. Their resemblances to the arachnids he attributed to convergence. He sought to establish a distant relationship with the Onychophora and the worms through two main hypothetical branches, the Vermacarina (Eriophyidae and Demodicidae) and the Protacarina (the remainder of the mites). He based his conclusions on the distinctive features of chelicerae, apodemes, pseudostigmatic organs, genital apertures, and the six-legged larvae.

According to André & Lamy (1), Haller and Canestrini considered the Acarina as an autonomous evolutionary class. The former authors contended, however, that chaetotaxy, segmentation, and other details of acarine morphology are difficult to resolve in phylogeny, but they concluded that the mites are strong examples of convergence and adaptation, with numerous peculiarities to differentiate them from the other arachnids.

In a discussion of fossil arachnids, Petrunkevitch (58) indicated that external characters alone are not enough to support a "natural classification." He insisted that the fundamentals of anatomy, physiology, and embryology are necessary to "produce corroborative evidence" of relationship. He maintained that external fusion of the abdomen and cephalothorax was accompanied by marked modifications of the mouth parts, which led to the production of three orders, the Opiliones, the Acarina, and the Architarbi. In a later paper (59), he modified the arrangement to include the Pseudoscorpionida and Scorpionida in the Latigastra.

Because of their small size and delicate structure, mites are not found commonly as fossils, but they have been reported from Devonian, Carboniferous, and Tertiary formations. All of the principal families are represented in the Tertiary period, particularly in amber, and the specimens show morphological features that demonstrate kinship with modern genera. Fossils of Mesostigmata, Ixodides, Trombidiformes, and Sarcoptiformes exist. The most ancient form is *Protacarus crani* Hirst from the Devonian sandstone of Aberdeenshire, Scotland. André & Lamy (1) indicated that this form is too well differentiated to be the common ancestor for the different groups of mites. They and Trägårdh (67) contemplated that this fossil might well be a

relative of *Palaeacarus* and lie between the Trombidiformes and Sarcoptiformes.

André & Lamy (1) and Grandjean (25) considered a polyphyletic origin for the Acarina because of the diversity of respiratory organs, and other features and also because the Mesostigmata and Ixodides are inherently different from the other suborders. André & Lamy (1) proposed that, possibly, the Mesostigmata (and Metastigmata = Ixodides) may have descended from the Opiliones. Vitzthum (68) seems to have concurred and explained a polyphyletic origin on the basis of the respiratory organs. Baker & Wharton (4) indicated that the Acarina are typical chelicerates whose ancestors were unknown and whose phylogenetic affinities were obscure and, further, that "most students of the group consider them to be polyphyletic in origin."

Petrunkevitch (58) concluded that, because of the three-jointed chelicerae, six-jointed palps with claws, spiracles on the second sternite, and the position of the genital opening between the last coxae in Opiliones, they and the Acarina had similar, inherent, and distinctive modifications. It is his opinion that these were attributable to creative trends within the groups and that the mites are most closely related to the Opiliones.

According to André & Lamy (1), Oudemans (57) attempted to make the Mesostigmata closer relatives of the Crustacea and designated the name Thrombidi-Sarcoptiformes for the single group of true acarines related to the Arachnida. In an earlier writing, Oudemans (50) noted that the caeculids were similar to the Opiliones because of the stiff setae on the legs and the sclerotization of the body, but he attributed this to convergence.

Snodgrass (64) indicated that, while the mites and ticks do not resemble other arachnids in appearance, they do demonstrate fundamental arachnid features in both external and internal morphology. He cited the chelicerae, palps, and four pairs of legs as examples and indicated that the acarines were arachnids that had "developed a highly specialized type of structure, mostly correlated with parasitic habits." He and Baker & Wharton (4) considered the gnathosoma (=capitulum), the absence of a prosomal-opisthosomal constriction, and the undivided prosomal region as distinctive features of the acarines.

No other arachnid exhibits a head structure comparable to the gnathosoma of the mites and ticks, and Snodgrass (64) indicated that the evolution of this structure from ordinary arachnid morphology is difficult to understand. He postulated, however, that the probable development included a dorsal and distal enlargement or extension of the pedipalpal coxal plates in a structure such as is found in the notostigmatid *Opilioacarus* With. By secondary sclerotization, such a dorsal extension would form the annular *basis capituli* and rostrum (=*tectum capituli*), distally the hypostome, all of which are typical, recent features of acarines.

From a general standpoint of arachnid relationship, Petrunkevitch (58) considered that the mouth parts of the acarines were modified by food and mode of life and that the homology of the parts was obscure. Gorirossi &

Wharton (20) partially clarified the homologies, and Hughes (41) considered that the mites as a whole had departed from the arachnid habit of active predation.

Petrunkevitch (58) concluded that the acarines had lost all traces of segmentation because the abdomen was not subdivided and lacked evidences of intersegmental lines. He also assumed that changes evolved in the prosomatic and opisthosomatic appendages but that they were lower than ordinal in character, e.g., Tetrapodili and parasitic mites. He also concluded that the nervous system of the acarines had undergone a forward movement of the neuromeres in contrast to the other arachnids, which may be evidence of a recent specialization, but more extensive data are needed to substantiate this conclusion.

The generally assumed phylogenetic relationships of the Acarina and other arachnids are indicated in Figure 1.

## Ordinal Relationships

Oudemans (50) considered the free-living, predaceous mites with tracheae, heart, and chelate chelicerae to be older than parasitic or plant-feeding mites that have modified chelicerae and lack heart and tracheae. He further concluded that the Mesostigmata, the Spelaeorhynchidae, and Ixodides are closely related because of the stigmata and genital openings. He also related groups of Trombidiformes (Prostigmata, Trombidiidae, Hydrachnellae, Halacaridae) by reason of the dorsal gnathosomal stigmata and considered them more recent than the Mesostigmata. He admitted exceptions regarding chelate chelicerae and types of genital openings and considered the Caeculidae similar to Opiliones because of convergence.

Among other features, Baker & Wharton (4) delineated three types of digestive systems correlated with the major suborders, viz., Onychopalpida-Mesostigmata-Ixodides, Trombidiformes, and Sarcoptiformes. They showed that the sarcoptiform type is more nearly like the mesostigmatid form than the trombidiform type.

Thor (65) eliminated the Opilioacaridae or Notostigmata from the main group of Acarina because of their anatomy and because he regarded their inclusion as purely artificial. He assumed a high degree of development for the Holothyroidea by reason of their four-part chelicerae, body sclerotization, heart, and reproductive organs.

Authors who assumed arachnid descent for the mites generally placed the Opilioacaridae intermediate between the Opiliones and the Acarina proper because of primitive characters supposedly retained from their ancestry (e.g., 10 segments of abdomen, with dorsal stigmata on four anterior segments, and newly formed tracheae tracheal system).

According to several authors (4, 68, 71), the Notostigmata are distinctly segmented mites with free coxae and four pairs of stigmata on the dorsal surface of the opisthosoma. Hughes (41) assumed a cosmopolitan distribution of these mites and considered that the hysterosoma of the mites is in-

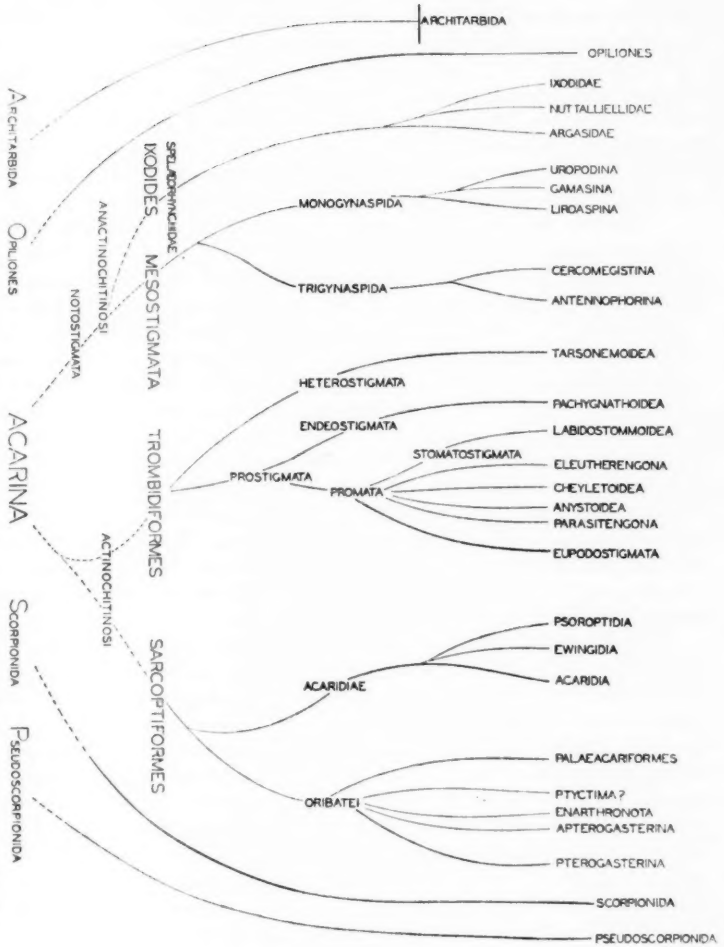


Fig. 1. A schematic representation of the possible phylogeny of mites adapted after various authors.

distinctly subdivided dorsally into 12 "segments," of which 10 belong to the opisthosoma. Baker & Wharton (4) indicated that these are primitive mites that possibly represent "living remnants of a former extensive fauna of primitive mites." Even though these mites are considered primitive, they exhibit morphological specializations in the possession of an epipharynx, secondary segments of the legs, and a divided tritosternum.

Camin, Clark & Bourdeau (10) and Grandjean (36) suggested that the notostigmatid Opilioacaridae have a primitive nature. Camin *et al.* assumed that these forms "represent a primitive offshoot of an original stock that perhaps gave rise to both the suborder Mesostigmata and the suborder Ixodides (=Metastigmata), but that interesting problem needs further clarification."

They also established the relationship of Opilioacaridae and Mesostigmata by an ingenious study of the palpal claw and tined setae. They found that the tined setae are actually reduced claws, as demonstrated by muscle attachments and other features. They suggested that the palpal claws migrated from the tip in Onychopalpida to the base of the palpal tarsus, where the claws became reduced to one claw of each pair or fused, and, further, that the reduced claw or fused claw persisted in the Mesostigmata as a "tined seta" and is a reduced claw homologous to the palpal claw of the Onychopalpida.

Grandjean (24, 26) suggested a relationship for the mites based on the presence of birefringent chitin in some setae and the ends of the chelicerae of the Actinochitinosi (Trombidiformes, Sarcoptiformes), and the lack of this actinochitin in the Anactinochitinosi (Notostigmata, Holothyroidea, Mesostigmata, Ixodides). Hughes (41) assumed that this arrangement represents evidence of a sound genetic relationship and followed this classification in part, as did Evans (18).

Mitchell (45) assumed that the primitive mites were all small and soft-bodied and suggested that considerable evolution occurred while they were still soft-bodied. His principal comments on phylogeny were applied to the water mites and will be discussed elsewhere in this paper.

André & Lamy (1) indicated that the relationship of the Opilioacarina and the Holothyroidea with the Mesostigmata cannot be ignored, even though they considered the former two autonomous, independent groups. They assumed that isolation in habits influenced their development and cited Lameere (44) as concurring in this opinion.

Grandjean (27) believed that, though primitive in certain respects, the Opilioacarina are too specialized in others to be the ancestors of the acarines. He recognized characters in the Opilioacarina that were common in both the Anactinochitinosi and some Actinochitinosi (notably the Prostigmata).

*Mesostigmata.*—The review by Camin & Gorirossi (9) explained very well the relationships and phylogeny of the Mesostigmata and should be consulted for details. They cited Trägårdh's postulations of a hypothetical ancestor of these mites, which was assumed to have had an elongated body

that was relatively soft and unsclerotized, with evidence of segmentation. The legs and gnathosoma were thought to be sclerotized, and it was assumed that the legs each had a sclerotized coxal plate with a single "sternal" seta and a lyriform "sternal" pore between the legs. It was postulated that the genital aperture was a flexible transverse slit without a cover, located immediately posterior to coxae IV. The epigynial shield presumably developed later.

Camin & Gorirossi indicated that the modern evidence discernible in female mites concurred with Trägårdh's postulations of the hypothetical ancestor, but they changed some of the taxonomic and morphological relationships on the basis of new evidence, as will be shown. They also assumed that the Opilioacaridae in the Onychopalpida are possibly very close to the hypothetical "proto-mesostigmatid."

They speculated on the probable development of the different subdivisions of the Mesostigmata and assumed that the bodies of the ancestral form foreshortened and lost nearly all evidences of external segmentation. A forward migration of the female genital slit occurred concurrently with a coalescence of coxal plates in different ways to produce the variations in jugular, sternal, and metasternal shields. The persistence of the latter was assumed to be a relatively primitive character. (Males were not considered extensively in the comparisons.)

Camin & Gorirossi (9) analyzed the genital shields and devised a system of classification and relationship for the Mesostigmata. This is a logical modification of Trägårdh's system and demonstrates a feasible phylogeny. In the Monogynaspida are those mites that exhibit the primary epigynial shield or that are derived from forms with a single genital cover. The Trigynaspida comprises mites with a mesogynial and two latigynial shields, including the mites for which morphological evidence indicates such shields are present, although fused in several ways. The sternogynial shield is also considered distinctive for this group.

In their re-evaluation of the Mesostigmata, Camin & Gorirossi (9) studied the sternogenital region of the female mites and, by dissection and comparison, obtained a much clearer understanding of the relationships of these shields than Trägårdh's representation. They determined three arrangements of the shields; viz., a single shield hinged posteriorly and opening outward like a trap-door (Machrochelidae), a mesogynial and two latigynial shields (Diplogyniidae), and a fourth, median, sternogynial shield that developed secondarily from a double origin (Fedrizziidae, Megisthanidae). The latter shield opens inward in these mites and is more heavily sclerotized than the epigynial shield of the gamasids and the uropodines.

It was postulated that the different kinds of genital plates were formed as progressive sclerotization of the body occurred, especially around the genital aperture, and that the genital opening became more rigid. Genital shields were produced between coxae III and IV and were relatively flexible at first. Later, the increasing sclerotization made them more rigid, and they were

fused by sutures to the ventral body wall. Those mites that developed the single epigynial shield became the progenitors of the Monogynaspida, where increased sclerotization accompanied a hardening of the exposed dorsal wall of the vagina to produce the median shield of the uropodines and gamasines.

Ancestral Trigynaspida possessed the basic mesogynial and two latigynial shields, in which more extensive modifications occurred, although the subgroups resemble each other more than do those of Monogynaspida. Enlargements, reductions, fusions, and alterations of the shields occurred partly because of the limited space of the shields and the compression of the ventral area.

In the trigynaspids, muscles attached from the anterior sternal region to the claviform vaginal sclerites pulled the dorsal wall of the vagina inward. These attachments, accompanied by sclerotization, formed the sternogynial shield. The vaginal sclerites probably became incorporated in the sclerotization of the shield and the muscles of the vaginal sclerites inserted on the shield itself after fusion, which resulted in a shield without sclerites that could be pulled inward. The "lost" sclerites were actually incorporated in the shield.

Another concept of shield development and phylogeny of the Mesostigmata was presented by Hughes (41), who conceived that the "nymphae," small subepigynial plates in the dorsal wall of the vagina, became the latigynial shields and were homologous to the vaginal sclerites of the trigynaspids. It has been questioned, however, whether these plates could make the transition from a dorsal position in the vaginal wall to the ventral site of the latigynial shields (personal communication with J. H. Camin).

Hirschmann (37) proposed a system of relationship for some of the Mesostigmata based on setal arrangements, but the system seems too complicated for practicality. A paper by him on the mouth parts (38) is unavailable to the writer.

André & Lamy (1) considered the acarines polyphyletic because the Mesostigmata and the Ixodides (= Metastigmata) are clearly distinguishable by inherent characters. They believed this indicated that the ontogenesis and ancestral forms were separated by a long time. Thor (65) also separated and established the Mesostigmata and Ixodides on the basis of a long, autonomous evolution. The Mesostigmata he derived from the hypothetical Urastigmata and insisted on a marked difference between them and other Acarina.

André & Lamy (1) concluded that the coalescence of the protecting dorsal shields in the Mesostigmata is a recent character and that the separation of the shields, as well as segmentation, is more primitive. They further assumed that Mesostigmata are quite different from the other acarines, because of the position of the stigmata, and, therefore, have weak affinities with them. They concluded that the long, straight peritremes in the majority of the Mesostigmata represents a primitive condition and traced the development through the undulating type in the uropodids to the recent or specialized

condition where the peritremes are short or lacking. They and Thor (65) also concluded that the nymphal development demonstrates stages that are similar to the hypopi of some Sarcoptiformes (=Atrachaeta). They cited an adaptive feature indicated by Comstock (11): the embryo has eight legs, as found in the nymphs and adults, but the fourth pair is resorbed before the birth of the larva and develops anew during the transformation of the nymph.

Thor (65) felt that the integument and shields are important in the determination of phylogeny. He proposed *Palaeogamasus* as the hypothetical, primitive common ancestor between the two main branches. He concluded that the Mesostigmata are clearly monophyletic in origin, that they exhibit differentiation, and that parasitic species are highly specialized. He assumed that there are four main branches from the common ancestor, viz., Uroprotosejidae, Uriphiopsidae, Urogamasidae, and Palaeouropodidae. The Laelaptidae and Iphiopsidae were primitive, the gamasids most highly evolved, but most other authors thought the laelaptids stemmed from the gamasids through modification and specialization from free-living forms.

Trägårdh (66) said that the evolution of the Uropodina (=Parastigmata) corresponds, in general, with that of the gamasids, as evidenced by the position of the genital opening. He indicated that uropodids are specialized with a retractible gnathosoma, foveolae pedales, and styloprocts, which are secondary modifications.

According to André & Lamy (1), several authors believed that the argasid ticks were derived directly from the uropodids and the other ticks from gamasid ancestors. They considered the position of the stigmata behind coxae IV an important and reasonable characteristic of relationship between the Ixodides and the Mesostigmata. They stated that, while the stigmata are so placed in Ixodides, ticks are unlike the Mesostigmata in the specialization of the large porous or granular stigmatal plates. (Argasids, however, have stigmata anterior to coxae IV, like the Mesostigmata.) In ixodids, the differentiation of immobile coxae was also considered distinctive.

Vitzthum (68) assumed that *Spelaeorhynchus* might be the link between the Ricinulei and the Mesostigmata, but André & Lamy (1) and Fonseca (19) concluded that the genus was closer to the Ixodidae. Several authors (1, 4, 5, 41, 48, 49) concurred in the opinion that the *Spelaeorhynchidae* are closely related to the ticks. These aberrant bat parasites obviously have mesostigmatid affinities, although the shape of the mouth parts resembles ticks, and Fonseca (19) considered them closer to the Ixodidae.

*Ixodides.*—The suborder Ixodides (=Metastigmata, =Ixodoidea) comprises the ticks. Members of the Argasidae are the leathery or "soft ticks," devoid of a scutum, and with stigmata anterior to leg IV, as in most Mesostigmata but with small spiracular plates. The Ixodidae are the "hard ticks" because of a scutum in all stages.

Oudemans (50) assumed that the argasids, with cylindrical palps and less modified body structures, are primitive and that the ixodids, with sheath-

shaped palps are more specialized and recent. The rare Nuttalliellidae are assumed by authors (4, 6, 50, 61) to be the missing link between the other two families because a bare outline of a scutum is present but not differentiated from the leathery and papillated integument. The mouth parts are anterior and visible from above, as in Ixodidae, but the palpal segments are movable, which is similar to the Argasidae. There is also an inner groove on the second palpal segment in Nuttalliellidae which Hoogstraal (39) assumed to be suggestive of the reduction of the terminal palpal segment to a small appendage of the penultimate segment in Ixodidae.

Both Mesostigmata and Ixodides exhibit a single pair of stigmata; in the ticks, however, the stigmata are usually posterior to coxae IV, there is no peritreme, and a large stigmatal plate is developed. The chelicerae and hypostome of ticks have been modified also because of parasitism.

André & Lamy (1) cited and partly concurred in the opinion of Schulze (62) that the Ixodides exhibit a relationship with the Anthracomarti from Devonian and Carboniferous times. They based their opinions on the ventral posterior furrow of the body in the ticks. Schulze (62) evidently derived the ticks directly from the Anthracomarti, which became extinct in the Carboniferous. He thought that the ticks became a lateral branch composed of parasites that may have had Permian reptiles as hosts. André & Lamy (1) mentioned the description of *Ixodes tertarius* Scudder from Oligocene rocks of Green River, Wyoming, but considered it a questionable citation. Petrunkevitch (58) was surprised that the Ixodidae were not found in the Paleozoic.

The argasid ticks were assumed to be descendants of uropodids and the other ticks of gamasids, but André & Lamy (1) considered such opinions forced, even though they accepted as intimate a kinship between Ixodides and Mesostigmata as between the other suborders. They concluded that the stigmata and other common characters were indicative of a primitive connection but considered them two distinct groups because of recent differentiation. The most noticeable difference they assigned to the mouth parts, and they cited some other features of uncertain importance, such as immobile coxae, slightly developed heart, and eyes in some tick genera.

Thor (65) admitted a possible common ancestor for the Mesostigmata and Ixodides in his hypothetical Hypostigmata, but he believed that the two groups detached from the acarine trunk well in advance of the Atracheata. According to André & Lamy, Oudemans (57) concurred in the idea of distinct separation.

André & Lamy (1) considered the Argasidae to be less evolved than Ixodidae on the basis of the simple, primitive palps and the relative lack of sexual dimorphism. They cited Bonnet (8), in whose opinion *Ornithodoros* was related to the Ixodidae and possibly the transitional form between *Ixodes* and *Argas*.

Several authors (1, 5, 17, 19, 68) assumed that the genus *Spelaeorhynchus* represented the closest link between the Mesostigmata and the Ixodides.

Thor (65) admitted a recent differentiation for the genus but assumed a probable separate evolution for these aberrant mites.

It seems feasible that the Ixodides developed from a mesostigmatid type of ancestor with the subsequent complete loss of the peritreme, the posterior migration of the stigmata (= Metastigmata), and the development of the large porous or granular stigmatal plate. Additional changes involved the development of the scutum in the hard ticks. The Argasidae are less evolved and closer to the Mesostigmata than the Ixodidae, and the Nuttalliellidae are intermediate. Undoubtedly, the development of exclusive parasitic habits by the ticks obscures some of the features of kinship.

*Trombidiformes.*—These are soft-bodied mites that sometimes exhibit an evidence of primitive segmentation in transverse rows of body setae, stigmata on or near the gnathosoma, and tracheae. The palpi are usually free; some have simple palpi, others have highly developed ones with a thumb-claw complex. The chelicerae may be chelate but are usually modified for piercing. Tarsi of the legs possess rodlike sensory setae, claws, and a padlike or clawlike empodium. Characteristically, some setae demonstrate actinochitin under polarized light, which Grandjean (25) assumed to be an inheritable feature relating them to the Sarcoptiformes in the Actinochitinosi.

Cunliffe (12, 13) considered the classification of the Trombidiformes and, in the former writing, attempted to arrange them phylogenetically. He compared several features, including tracheae, palpi, chelicerae, body setation, genitalia, and tarsal structures, and used these to ascertain the taxonomy and, in part, the phylogeny of these mites. These features and specializations in certain groups led him and Thor (65) to consider the grouping above the family level as reflections of natural relationships. Most of the names and relationships used by Cunliffe (12) are generally accepted by the majority of workers in the field. He arranged the mites into two major groups, one with simple palpi, the other with a thumb-claw complex. In the latter condition, the distal segment of the palp has migrated ventrally to become the "thumb," and the tibia has developed a claw or strong seta. This arrangement is sometimes absent, but other common features demonstrate general kinships.

Simple palpi usually occur in mites without peritremes, but there are exceptions (Paratydeidae). In mites with the thumb-claw complex, peritremes normally accompany the modified palp. Of this latter complex (thumb-claw and peritreme), there may be few body setae arranged in transverse rows (Promata, Eleutherengona) or many setae (Parasitenini, Parasitengona), though exceptions do occur.

Cunliffe (12) considered the chelicerae the most important feature for assessing relationships in this suborder. He concluded that the opposed chelate chelicerae were primitive and typical of the Eupodostigmata, Endeostigmata, and Stomatostigmata. The more highly developed mites, mainly predators and plant feeders, had degenerated the fixed chela, accompanied

by an elongation of the movable digit into a piercing stylet or needle, which is straight and protrusible in the Erythraeoidea and recurved and whiplike in the Tetranychoidea. Smaridiidae exhibit an extensive specialization in that the entire gnathosoma may be withdrawn.

Typically, the genital aperture in trombidiforms is a longitudinal slit with or without suckers. Tetranychoids and eriophyoids are exceptions, however, with their transverse slits. Mites with simple palpi usually exhibit little sexual dimorphism, although the males have smaller bodies and genital apertures but greatly enlarged tarsal sensory setae used for (?) detection and contact. Some mites of the thumb-claw complex (Tetranychoidea, Raphignathoidea, Cheyletoidea) are dimorphic. Males are smaller than females, have longer legs, larger sensory setae, and a strongly sclerotized aedeagus. Heteromorphic larvae are complex and unclarified.

Tarsal claws, empodia, tarsal sensory setae, and chaetotaxy were considered by various authors to be important at specific and higher levels (12, 31, 47, 70). Cunliffe (12) found that tarsal patterns may be typical for superfamilies, where tarsal claws, empodia, and sensory setae are basically of the same type. Tetranychoids exhibit a set pattern of claws and pulvilli for each family. He also regarded body setation as significant and believed that setae in transverse rows constitute a primitive condition and reflect previous segmentation. He indicated that the larvae of the densely hirsute Parasitenini have a primitive pattern and show a basic relationship to the other groups. He also predicted some aspects of habitat from morphology; e.g., chelicerae = predaceous (Raphignathidae, Tydeidae); lack of rodlike sensory setae = aquatic (Halacaridae, Hydrachnellae).

If one can assume that classification indicates some measure of relationship on a morphological basis, the Trombidiformes, as described by Cunliffe (13), must be considered indeterminate forms in some respects—a concept at variance with the opinions of other authors (1, 36, 41). The works of both Cunliffe (13) and André & Lamy (1) are extremely useful for current considerations.

The Heterostigmata, which comprises the Tarsonemoidea, are considered basic stock or near the early ancestors of the Trombidi-Sarcoptiformes complex. Lameere (44) assumed that these mites were too strongly transformed by parasitism and other causes to allow differentiation of truly primitive characters and those recently acquired. André & Lamy (1) indicated that the Tarsonemoidea have been related to the Oribatei by some acarologists and to the Cheyletidae by others but that more recent authors believed they had affinities with the Acaridia (= Tyroglyphidae) and, possibly, a common origin. Cunliffe (12) concurred in the latter opinion because trombidiforms are distinguished by a membranous pulvillus, which may indicate a positive affinity with the sarcoptoids. He included the Podapolipodidae, Scutacaridae, Pyemotidae, and Tarsonemidae in the Heterostigmata.

André & Lamy (1) assumed that trombidiforms showed regression of legs

and some body parts in their evolution. They believed the Devonian *Protacarus crani* resembled *Eupodes* or a similar Prostigmatid. They also assumed three main groups and, with Thor (65), placed the Labidostommidae near the stem of the primitive Prostigmata, although other authors linked these with the Oribatei.

Grandjean (26) related the Pachygnathidae to the inferior Oribatei (Hypochthoniidae) because of the segmentation of the hysterosoma, which he considered an index of ancient relationship.

Prostigmatids possess stigmata on the anteromedian dorsum of the gnathosoma between the chelicerae, from which extend unlined and unbranched tracheal trunks. Thor (65) cited the exceptionally numerous branched and lined tracheae in the Trombiculidae as evidence of an advanced stage of development, and he admitted a possible monophyletic origin for the Prostigmata and Stomatostigmata in the hypothetical *Urotydeus*. A possible kinship with the atracheate Acaridiae (=Sarcoptoidea) and Oribatei was construed by André & Lamy (1) on the basis of the chelate chelicerae in the Prostigmata.

The latter writers concluded that the presence of pseudostigmatic organs in Heterostigmata, in a few Prostigmata, in Acaridiae (=Atracheata), and in the Oribatei was of phylogenetic importance, even though these sensillae were modified into cristae metopicae in some Trombidiformes. They also used apodemes, legs and claws, excretory organs, and ventral genital suckers as evidence of kinship.

Thor (65) believed that the presence of four pairs of genital suckers in some Prostigmata indicates common affinities of Eupodidae, Rhagidiidae, and Smaridiidae. In others, with three pairs of suckers, Pachygnathidae, Bdellidae, and Trombidiidae, he assumed a closer relationship with the Oribatei. He also was convinced that the Ereynetidae, Tydeidae, and Eupodidae were relatively highly developed Prostigmata because of the genital suckers and sensory hairs.

André & Lamy (1) and Cunliffe (12) believed that the Eleutherengona comprised the Bdellidae, Cheyletidae, and Tetranychidae, the Demodicidae arose from the Cheyletidae, and the Eriophyidae from the Tetranychidae, and that all were from the basic stock of the Eleutherengona. André & Lamy (1) thought that resemblances in families of different trombidiforms might be attributable to convergence.

The Demodicidae, specialized, vermiform endoparasites in mammalian hair follicles, possess four pairs of rudimentary legs, but neither stigmata nor tracheae. They were first regarded by Ewing (15) as degenerate, but ancient, Sarcoptiformes. He later (16) agreed with other authors and assumed that they were of cheyletid stock because they lack an anus and have a dorsal genital orifice in the male. Thor (65) believed that they are more closely related to the Prostigmata and the Eriophyidae because of the annulated integument, simple mouth parts, short legs, and absence of tracheae

and stigmata. In his conception, their position was part of the basic vermiform acarine stock derived from his Uracarina as two separate and distinct lines.

André & Lamy (1) concluded that the similarity of body form and integument was attributable to convergence resulting from degraded specialization and parasitism, the Eriophyidae being more highly evolved than the Demodicidae. They and Cunliffe (12, 13) concluded that the Demodicidae were cheyletoid mites. The latter author related them to the Myobiidae and Cheyletidae because of the gnathosoma, claws and pulvilli, and the adeagi. The former authors also discussed the origin of vermiform acarines in other suborders (e.g., Halarachnidae, Listrophoridae) on a similar basis of convergence, specialization, and parasitism, but Thor (65) considered the vermiform acarina primitive.

The wormlike, gall-producing Eriophyidae are assumed to be highly specialized by most authors, although Schulze (62) said that they are neotenous. André & Lamy (1) considered the Phyllocoptidae a young branch of the Eriophyidae, but Baker & Wharton (4) followed Kiefer's composite grouping of a single family.

André & Lamy (1) cited the considerations of Dahl (14) and Banks (5), who related these mites to some of the Tarsonemoidea that infect and deform plant parts. The former authors concurred with Ewing (16), who established their derivation from the Tetranychidae. Oudemans (51) thought that the eriophyids originated from arboricolous Trombidiidae but changed his opinion (53) to the tetranychid relationship. André & Lamy (1) and Cunliffe (12) corroborated this relationship because the mouthparts were like cheyletoid-tetranychoid stock. Considering the legs, tarsi, and empodia, they refuted the possibility that the Eriophyidae had a sarcoptiform relationship. Hughes (41) arranged the Eriophyidae in a separate suborder, Tetrapodili, but indicated that they may be considered in the Trombidiformes.

Oudemans (54) arranged the Parasitengona on the basis of stigmata and tracheae. Cunliffe (12, 13) made a revision of the cohors under a variety of characters, including the presence of a well-developed palpal thumb-claw, many setae, usually a crista metopica, tactile and sensory leg setae, tarsal claws, and absence of extreme modification of the empodium. All of these mites possess heteromorphic larvae.

The Halacaridae (= Pleuromerengona) were separated by various authors because they possess neither stigmata nor tracheae. According to André & Lamy (1), some authors related these marine mites to the Hydrachnellae by a narrow kinship, but André & Lamy and Cunliffe (12, 13) concluded that halacarids are not related to the water mites. The former authors (1) and Vitzthum (68) concluded that they perhaps split off at an early epoch. Evidently the Halacaridae are much more closely related to the Eupodostigmata than to the Eleutherengona and are "sea-going" cunaxids, as determined by their cunaxoid mouthparts and other general structures. They are mostly carnivorous, although a phytophagous, littoral species was

cited by André & Lamy (1), who indicated that the palp in these mites was observed by various authors to be bifid or branched, to resemble a small chela, and, supposedly, to function as an antenna.

Systematic unity is not a characteristic of the Hydrachnellae, or water mites, although several authors arranged them into three tribes. André & Lamy (1) and Mitchell (45) admitted the difficulties of determining the phylogeny. The former authors indicated that these mites were manifestly derived from terrestrial trombidiforms and had conserved a similar arrangement of internal organs and the same mode of tracheal respiration. They believed that the Hydrachnellae (=Hydracarina) do not represent a firm biological group and appear to have affinities with several families.

Vitzthum (68) postulated a diphyletic origin for them, while André & Lamy (1) assumed a multiplicity of ancestors. Thor (65) originally thought that the water mites arose from several fundamental separate trunks, but evidently amended his conclusion by a study of the palps to one of a monophyletic origin. He found an affinity with the Trombidioidea in the distal palpal digits and in certain hairs. André & Lamy (1) also considered the presence of a vestigial pseudostigmatic organ in some adult hydracarines an important index of regressive trombidiform relationship. The reduction of the size and function of the organ was attributed to the aquatic life as contrasted with the well-developed organs in terrestrial forms. Genital suckers were also used by these authors as a basis for relationship, but variations in the suckers and accompanying structures hampered the analysis and the clarity of the conclusions.

Evidence points to terrestrial and polyphyletic ancestors for the Hydrachnellae; in this respect, André & Lamy (1) referred to the conclusion of Motas (46) that the immature stages of water mites reflected this connection in their phoretic and parasitic habits and in modifications that had occurred over a very long period. The paludal forms, incapable of swimming and walking, were considered recent. The morphological variations, e.g., podal and apodemal changes, sclerotization, and increase in suckers, were thought to be recent specializations determined by adaptations to environment. A gradual transition of forms was postulated from the land to moist moss to semiaquatic to lotic and, finally, to limnetic environments, but this transition was not without exceptions.

Mitchell (45) indicated that the current arrangements of families and groups above the family level do not express phylogeny. He believed that the exoskeletons of mites do not exhibit homologous sclerites that indicate phylogeny but that the acquisition of sclerites and development of an exoskeleton is relatively recent in the evolution of mites. He indicated that the homologous segments of water mites were segments of appendages and had modified the sclerotization of the body for muscle attachments. This modification possibly occurred singly or in combinations in four major ways. Firstly, the basal leg sclerites encased the body; secondly, expansion of coxae or sclerotization of membranous areas resulted in swimming or walking forms;

thirdly, sclerotization altered the body form and shape in mites inhabiting swift streams; fourthly, thickening of extensive membranous areas occurred.

Mitchell (45) considered that the primitive water mites were unspecialized in locomotion and lacked sclerites except on the appendages. He believed that the ancestral form existed for a long time and gave rise to "15 or 20 stocks, each of which evolved a peculiar exoskeleton," and became "represented as a family or closely related group of families." He also concluded that the excessive number of families of water mites obscures the phylogeny. He observed that it is an erroneous practice to consider the exoskeleton as the only unstable morphological feature in the phylogeny of water mites or other acarines, and he substantiated this view by referring to Bader's (2) study of the mid-gut of water mites. He (45) concluded that studies of internal anatomy, life history, embryology, and behavioral traits, together with an understanding of the functions and skeletal development, are vital and basic to clarification of phylogeny. He indicated that, since the primitive mites were probably small and soft-bodied, they could have evolved extensively in this state, hence a study of the exoskeleton alone might produce erroneous concepts on phylogeny.

Regarding Trombidi-Sarcoptiformes relationships, André & Lamy (1) indicated that body setation and chelate chelicerae and similarities in coxae, empodia and claws, pseudostigmatic organs, and areae porosae indicate a close relationship between these suborders. Oudemans (56) considered the affinities so marked that he proposed to unite these mites into a single group, the Thrombidi-Sarcoptiformes. Grandjean (25) also corroborated the relationship with his discovery of actinochitin in the two groups.

Thor (65) derived these two major groups from the hypothetical Urastigmata through the intermediate transition of the hypothetical Protastigmata. He assumed that the Sarcoptiformes and Trombidiformes possibly developed through Protastigmata into two major branches, which differentiated into the ancestors of the Oribatei and Acaridiae (=Ventrostigmata) with or without ventral coxal stigmata and ancestors of the Trombidiformes (=Dorsostigmata) with dorsal gnathosomal stigmata.

André & Lamy (1) emphasized that the chelate chelicerae (with some cases of extreme modification) and similarities of palps, legs, apodemes, and genital suckers substantiate relationships between the two suborders. They assumed that evolution produced the present differentiated state of specialized forms. Accessory, modified, or rudimentary features were more recent, e.g., stylet-like chelicerae of Tetranychidae. They concluded that, in the inferior forms of Prostigmata, Heterostigmata, Acaridiae, and Oribatei, these structures were initially simple and that differentiation in many was relatively slow and fairly equal in extent. Thor (65) assumed that the original ancestral type was restored in the Acaridiae (=Atracheata).

Body sclerotization in the Trombidi-Sarcoptiformes relationship indicates all sorts of possible transitional arrangements and transitional forms. The pseudostigmatic organs, genital suckers, and similarities of nymphal forms seem to indicate a common origin for the Oribatei, Heterostigmata,

and Prostigmata but divorce the Acaridiae (=Atracheata). Connections may be assumed from Thor's (65) Urotracheata to Ventrostigmata to the Oribatei through the Hypochthoniidae. It is also possible that the Acaridiae lost the pseudostigmatic organ in the same way rodlike sensory setae were lost in water-dwelling trombidiforms or pseudostigmatic organs in some Oribatei (*Hydrozetes*).

André & Lamy (1) indicated that, generally, acarologists agreed that the coxae of primitive mites were free and secondarily became sunken and fixed with fused coxal plates or apodemes. Grandjean (28) proposed an inverse concept of fixed coxae and secondarily separated, mobile projections. If the latter is so, the Acaridiae are extremely primitive and represent ancient stock. In another paper (25), he related the Trombidiformes to the Sarcoptiformes in the Actinochitinosi because of actinochitin in some setae and the chelicerae (35).

*Sarcoptiformes.*—The ancestry of Sarcoptiformes is assumed to have been in free-living mites and to have given rise to the two major branches of the suborder, the Acaridiae and the Oribatei. The former show a primitive form, weak body sclerotization associated with a free life that is modified by parasitism in some, apodemes, and no stigmata or tracheae. The Oribatei demonstrate strong sclerotization, though not exclusively, hidden tracheae associated with the coxae (=Cryptostigmata), and, usually, prominent pseudostigmatic organs.

According to André & Lamy (1), Oudemans and Trouessart assumed that the skin was the primary agent of respiration in the Acaridiae and the tracheae were secondary developments. Tracheae were rarely observed in Acaridiae, according to André & Lamy (1), who attributed this to the possible absence of tracheae in the ancestors and also in current forms (29). The opposite view was taken by some authors: the tracheae were present in the ancestors but by regression became so fine that they are undetectable.

M. Hughes (40) considered the Sarcoptiformes the most specialized of the acarines, and André & Lamy (1) suggested that the hypopal stages of the Acaridiae were uniquely related within families of the Acaridiae and similar to like stages of Trombidiformes. They suggested that the sarcoptids developed from tyroglyphids with *Coleoglyphus* representing the transitional form between Tyroglyphidae and Canestriniidae and that Epidermoptidae was transitional between the itch and feather mites. They postulated that the tyroglyphids gave rise to predatory forms, which, in turn, may have been ancestral to some of the parasitic groups. Oudemans (52) considered those mites with free apodemata I to be the most primitive and sternal expansion and fusion to be later developments.

André & Lamy (1) pointed out that the specialization of parasitic species had produced even more striking modifications in the morphology and physiology. The vermiform Acaridiae were assumed to have had vermiform ancestors (65), but André & Lamy (1) attributed this feature to adaptation (e.g., Demodicidae, Eriophyidae, and Listrophoridae).

Thor (65) and Oudemans (55) assumed that the Acaridia were related to

Psoroptidia on the basis of hypopal or migratory nymphs. Thor (65) derived the Tyroglyphoidea (=Acaridia?) and the Psoroptoidea from ancient forms in which hypopi may have been present. His hypothetical Urastigmata was intermediate to Prostigmata. The main trunk, Urolistrophoridae, produced the elongated, annulated Listrophoridae and became the progenitors of the Uranalgesidae, from which arose the Psoroptoidea (Analgesidae, Sarcoptidae, Psoroptidae) with certain migratory nymphs resembling acaridian hypopi. From the other main trunk, Urotyroglyphoidea, stemmed the Anoetidae, Rhizoglyphidae (=Saproglyphidae of Yunker), and allied families. André & Lamy (1) admitted a strong tendency for variation in these two main groups and considered the Canestriniidae intermediate between them. Yunker (73) evidently agreed in part with the latter but placed the Ewingidae between the free-living cheese mites and the parasitic feather and itch mites. He based this relationship on the female genital apertures, genital suckers, genital apodemes, and tarsal modifications.

Oudemans (50) considered the free-living acarids older and the parasitic forms younger, with differentiation and specialization obviously developed more recently in the parasitic groups. The canestrinids he considered older than the more recent, degenerate listrophorids. The sarcoptids he assumed were more primitive because of the chelate chelicerae and transverse genital opening; the cytoditids were more recent. Yunker's (73) concept of relationship differed in several respects, and Zakhvatkin (74) and Robertson (60) proposed other changes, related principally to taxonomy.

The Oribatei (=Oribatoidea, =Octostigmata, =Cryptostigmata) are secondarily sclerotized Sarcoptiformes that resemble minute beetles and, hence, are referred to as beetle mites or moss mites because of their natural appearance and habitat. When stigmata are found in these mites, they are located in the thin acetabular membranes between the coxal and trochanteral segments of the legs. The epimera or coxal apodemes are usually sunken and plainly visible.

In the Oribatei there has been an obvious tendency for sclerotization, but in some of the primitive forms the integument is relatively soft and sometimes bears evidence of segmentation (Enarthronota). According to André & Lamy (1), there is a progression toward fusion from the Phthiracaridae, with a movable hinge or suture between the aspis and notogaster, through the apterogasterines with varying amounts of propodosmal-hysterosomal mobility, to the pterogasterines with pteromorphs. Oudemans (50) divided the oribatids on the basis of a more recent ability to close like a phthiracarid and the absence of this ability, which he considered more primitive. Most authors agree that the apex of specialization and development is in the Galumnidae, with no suture between the propodosoma and hysterosoma, and in highly developed pteromorphs. André & Lamy (1) concluded that, with sclerotization, the stigmata and tracheae were developed secondarily.

Palaeacariformes was erected as a new suborder by Trägårdh (67) to include two genera of mites that he considered primitive, *Archeonothrus* and

*Palaeacarus*. Because of the gnathosomal stigmata, he assumed them to be relatives of the Trombidiformes and further postulated that the Devonian *Protacarus crani* was not an ancient eupodid but a relative of the inferior oribatids. Grandjean (22) assumed that the characters of the Palaeacaridae, for which he described the new genera *Aphelacarus* and *Acaronychus* and included Berlese's *Parhypochthonius*, were related to the inferior Oribatei. The major exception was the cheliceral stigmata, which were more typical of the Prostigmata. The uncovered gnathosomal forms he called the Astegasima; the covered group (including the Aptyctima and Ptyctima) he termed the Stegasima (34).

Later, Grandjean (36) explained that common characters did not always come from the same inheritance or from the same adaptation to the environment and that multiple appearances of a character were independent of each other and were not rare in the Oribatei. He assumed evolution acted on many characters in many directions, and he divided the Oribatei into 11 natural groups based on nymphal and adult characters. He also summarized relationships and morphology of the primitive oribatids in other papers (30, 31, 33).

In another paper (23), Grandjean recognized a series of tracheal organs from the primitive forms through the intermediate types to the specialized oribatids. He postulated the development of these respiratory structures from a simple air pore through a stage of small pockets (brachytracheae) to the tubular type of tracheae. Since tracheal apparatus appeared to be lacking in the primitive or least evolved oribatids, e.g., Hypochthoniidae, Camisiidae, and in the majority of the nymphs, as well as in the Ptyctima, André & Lamy (1) assumed that the primitive (inferior) oribatids and young forms were related to the acarids and that the superior oribatids had a uniform tracheal system of later origin and development.

Jacot (42, 43) estimated that the Oribatei represented an array of diversely sclerotized relatives of the tyroglyphids and concurred with Thor (65) that the Hypochthoniidae represented the point of evolutionary departure of the oribatid mites. The hypothetical Urohypochthoniidae of Thor (65) was considered to have been ancestral to both the Aptyctima and the Ptyctima, but Jacot assumed that the Ptyctima had a separate origin. Grandjean's concept (31, 32, 33) of these basic forms differed in some respects.

André & Lamy (1) indicated that the numerous intermediate forms were unknown. They postulated that the basic patterns of sclerotization were indications of a relationship and concluded that special tendencies, structures, and anatomical features were difficult to assess and evaluate in terms of phylogeny. They concurred with other writers in the concept that the Galumnidae represented the apex or highest development of the Oribatei, which made the development of the pteromorphs a progressive feature of the Pterogasterina that culminated in the highly evolved Pelopidae and Galumnidae.

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Grandjean (28) considered the pseudostigmatic organs important in the understanding of phylogeny. He assumed that the reduction of these organs in some adults was regressive and not primitive. André & Lamy (1) pointed out that this loss in *Hydrozetes* was attributable to adaptation to the environment, and they compared it to regression of similar structures in Hydrachnellae.

Grandjean (21) also reported the changes in the modified chelicerae of the Oribatei, where forms like Gustaviidae have become specialized. The preliminary plan of oribatid relationship by Woolley (72) differs from Grandjean's (30, 31, 33, 36) arrangements, but, with additional investigations, these discrepancies are sure to be clarified, the phylogeny better understood.

## Summary

No known connections with other arachnids can be ascertained from the fossil record, but the chelicerae and other morphological features indicate that the Acarina probably had an arachnid origin in opilionid-like ancestors but developed with inherent peculiarities, principally the gnathosoma. They were at least diphyletic, probably polyphyletic, in origin, with two main groups, the older Anactinochitinosi (Notostigmata, Mesostigmata, Ixodides) and the younger Actinochitinosi (Trombidiformes, Sarcoptiformes). In general, the groups lost traces of segmentation, changed appendages, and compressed the neuromeres forward. Possibly the primitive Opilioacaridae are intermediate between the Opiliones and Mesostigmata or very close to the hypothetical "proto-mesostigmatid." However, Opilioacaridae and Holothyroidea may be autonomous, independent groups.

In the Mesostigmata, the Monogynaspida and Trigynaspida are differentiated by ventral and vaginal sclerotization. Those mesostigmatids with peritremes are the more primitive but are related to the parasitically modified Ixodides by Spelaeorhynchidae. The Argasidae are older and closer to Mesostigmata because of the stigmata, the Ixodidae are more recently specialized, and the Nuttalliellidae are intermediate between Argasidae and Ixodidae.

The chelicerae indicate relationships in the Trombidiformes with primitive chelate forms and the more recent, specialized piercing types. Tarsi and body setation also indicate relationships, the Heterostigmata being near the early ancestors of the suborder, which is probably related to the Sarcoptiformes by the pseudostigmatic organ (=crista metopica), genital suckers, coxae and apodemes, claws, body setation, nymphs, and actinochitin. Tarsi, chelicerae, and other features separate the Endeostigmata in relation to the Promata, from which stemmed the Stomatostigmata, Eupodostigmata, the Eleutherengona, and the Parasitenini. The Eriophyidae and Demodicidae represent specialized vermiform trombidiforms of two separate derivations. Halacaridae and Hydrachnellae are also specialized and perhaps of terrestrial ancestry, but their origin is obscure, possibly polyphyletic.

The Acaridiae of Sarcoptiformes may represent primitive stock that developed from free-living mites possibly devoid of tracheae and close to the stem of Trombidiformes. The Acaridia are more primitive, the Ewingidia intermediate, and the Psoroptidia more specialized and parasitic. The Palaeacaridae possibly have close affinities with the Prostigmata of Trombidiformes, because of cheliceral stigmata, and with the primitive Oribatei (e.g., Hypochthoniidae). The Apterogasterina or "unwinged," mainly nonanchylosed Oribatei are older, more primitive; the Pterogasterina with pteromorphs are more recent and illustrate a development through several types to the Galumnidae at the apex.

Phylogeny should be based on a critical review of morphology, embryology, physiology, and paleontology, but the relative paucity of these data for the acarines makes phylogenetic trees largely illusory.

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# PALAEOENTOMOLOGY[1]

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The vast amount of newly found locations of fossil insects (109) and the considerable increase in collections of insect remains make the study of whole fossil faunas a difficult task for one person. Only a few works of this kind were published during the past decade (15, 47, 48, 106, 107). Diversity of insects existed as early as in the Carboniferous period; palaeoentomologists therefore tend to specialize in separate groups of fossil insects, according to their interests and our knowledge of the recent fauna. This method of study increases our knowledge of the system of contemporary orders and enables us to better understand the fossil material.

Some 300 papers with descriptions of fossil insects have appeared in the last decade; however, there have been but few reviews or revisions of the separate orders. More recent reviews and surveys of fossil insects of all orders are essential; this is particularly true of the insects from Baltic amber, since the largest collection of insect-containing ambers (Koenigsberg University) did not perish during the Second World War and is now preserved in Berlin. The bulk of the literature published is devoted to descriptions of new forms of insects and to redescriptions of old ones.

*Apterygota.*—The finding of a large number of *Dasyleptus* remains in good condition in the Lower Permian deposits of the Kuznetsk basin, classified by Sharov (117 to 120) in the new order Monura, allowed the author to support the view of Brongniart on the peculiarity of *Dasyleptus* found for the first time in Stephanian of Western Europe.

*Palaeodictyopteroidea.*—Our knowledge of the order Palaeodictyopteroidea, the most ancient in the superorder, is sparse. Therefore, the finding and description of new species is of great importance and interest. The following works are devoted to this question: Demoulin (27, 37, 39), Kukalová (56, 58, 64), Laurentiaux *et al.* (66, 69, 71, 74, 75, 79), Richardson (100), Zázvočka (141). The figures illustrating the descriptions presented are not always accurate, and the systematic position of the described taxonomic unit is not always defined correctly. Haupt (46) presented a few reconstructions of some Palaeodictyoptera, but they are of no importance since no well-preserved factual material was available to the author. Reconstructions made up speculatively are often erroneous; they become engraved in one's memory and are an obstacle to the proper understanding of new material.

Demoulin (29, 39) revised the order Archodonata; he was right when stating new superfamilies and families for previously described species from the Upper Palaeozoic. Demoulin also revised fossil representatives of the

[1] The survey of the literature pertaining to this review was concluded in March, 1960.

order Ephemeroptera (25, 26, 28; 30 to 36; 38) and described again some of the old materials kept in European museums. Most of these materials are, unfortunately, in bad condition. Though this author almost totally lacked new collections, he succeeded in reinvestigating the genus *Mesephemera* and in proving the erroneousness of old figures on the basis of new data. The success of Demoulin's work is to a great extent attributable to his knowledge of contemporary Ephemeroptera. His new arrangement of the order may be disputed, but it seems to the reviewer that new material in good condition will show his system to be correct.

*Megasecopteroidea.*—Carpenter (20) revised the Carboniferous Megasecoptera of France. He found the path of evolution of this group toward costalization of the anterior wing margin, simplification of its venation, and narrowing of the wing basis, which conceals the run of longitudinal veins. It is likely that the fusion of the bases of all longitudinal veins into one stem is but an apparent one. The figures presented by Zalessky (140) in his work describing two species of the genus *Pseudohymen* give no generally acceptable interpretation of the wing venation of this order.

*Odonatoidea.*—The study of multiple fossil representatives of this superorder is a problem to be solved in the future. During recent years only a few works appeared in this area: three species of Meganisoptera-Meganeuridae, two species of the Ural Permian (129, 136, 139), and one species from the Triassic of France (44) have been described. Of interest is the finding of two Permian species of Protanisoptera—*Callimokaltania* and *Hemizygopteron* (= *Ditaxineura* Mart.)—in the Urals and Kuznetsk basin (138).

*Blattoidea.*—Descriptions of new species of the order Blattoidea are being accumulated, an exception is the revision of the Frič species undertaken by Kukalová (57). There have been no investigations on individual variability in venation of tegmina which could significantly reduce the number of the "species" described and thus make the cockroch system less artificial. The establishment of a clear-cut system of Blattoidea is of great importance; some orders (Rhynchota, Neuroptera, and others) seem to have originated from different branches of Blattoidea. It is very unlikely that all Pterygota originated from Archimylacridae alone. New forms have been described by the following authors: Brown (16), Kukalová (56), Laurentiaux (65, 68, 70), Laurentiaux & Teixeira (77), Müller (96), Zalessky (135, 136), and Zeuner (142). The finding of a Blattoidea with ovipositor (68, 135) is interesting but, unfortunately, its morphological structure has been inadequately analyzed.

Some new forms of Protoblattoidea from the Palaeozoic period in Europe have been described by Kukalová (61, 63) and Laurentiaux & Teixeira (76). Weidner (127), the specialist in recent Orthopteroidea, has undertaken a study of previously described Isoptera from Baltic amber and has presented a key to species identification. New species of Cenozoic termites are described in some works by Snyder (121 to 123).

*Orthopteroidea.*—Jeannel (53) divides this superorder into three orders,

namely Protorthoptera, Plecoptera, and Orthoptera; all Saltatoria are included in the latter. Carpenter (17) has established his own system: the order Protoperlaria is distinguished with a single family Lemmatophoridae, and all other fossil Orthopteroidea are referred to the order Protorthoptera. He accepts the previously established orders Caloneurodea and Glosselytrodea but does not regard Miomoptera as an independent order. In his work devoted to a description of the family Liomopteridae and of new families related to the former, Carpenter shows that Protoperlaria possessed an aquatic larva, whereas Protorthoptera possessed a terrestrial one. This diversity of opinion on the systematics of the group in question, which was voiced over a short period of time (1949–1953), demonstrates that the problem is a difficult and vague one. Only new findings of remains, not only of wings but of the bodies as well, will be able to solve this problem.

The reviewer thinks that Miomoptera is an actual order, as are Caloneurodea and Glosselytrodea. It seems necessary to separate Orthoptera-Saltatoria, in which fossils are also included, into a special group and to classify the remaining cursorial forms into another order. New species belonging to the following families have been described: Ideliidae (105), Sthenaropodidae (105), Cacurgidae (67, 100), and Lemmatophoridae (19, 132, 134). In Saltatoria the following have been described: Oedischiidae (62, 78, 137), Paoliidae (50, 60, 62), Tcholmanvissiidae (131), Tettigoniidae (103, 128), Haglidae (83), and Prophalangopsidae (105). Having studied the morphology of the ovipositor in Permian *Tcholmanvissia*, Machotin (81, 82) pointed out phylogenetic relations of some groups of Saltatoria.

*Plecoptera.*—Fossil stoneflies have been studied very little recently and we have only old descriptions, despite the fact that this order is a very ancient one and closely related to some Permian Protoperlaria. The finding of the new mid-Jurassic fauna in the Kuznetsk basin in which stonefly larvae of *Mesoleuctra gracilis* (80), previously known from Ust-Baley (eastern Siberia), occurred along with the species of Odonata and Homoptera.

*Miomoptera.*—Both the existence of this order and its relationship—whether it belongs to Psocopteroidea or is more closely related to Orthopteroidea—is a disputable problem. Martynova accepts the independence of this order and includes in its composition two families only, i.e., Archaemiopteridae, of which she has described a new representative from the Upper Permian of the Kuznetsk basin (93), and Palaeomantoidae, to which belongs almost the bulk of Miomoptera known to us. The new genus of the latter family, *Miomatoneurella*, which is very similar to Embioptera by the venation character, is described by Martynova in the same paper. Of great interest is the finding of two larvae in lower Permian layers of the Kuznetsk basin; having studied venation of their nymphal wings, Sharov (116) classified these larvae as belonging to the order Miomoptera, probably to the genus *Delopterum*.

*Embioptera.*—In the Permian deposits of Europe, a representative of embiids, *Sheimia*, was found and described for the first time by Martynova

(93). Zalessky (130) may be right when thinking that Embioptera originated from some cursorial Orthopteroidea of the *Tillyardembia* type.

*Glosselytrodea.*—Having reinvestigated wing venation of Permian Jurinidae, Martynova (93) referred to them also the new family Euglosselytridae, which she had studied earlier (87). On the basis of new material, she stated that wings in the genus *Anorthoneura* are the hind wings of the genus *Jurina*. A set of new species from different horizons of Permian deposits in the Kuznetsk basin has been described, and the part played by them as stratigraphic indices has been pointed out. The family Permoberothidae, previously classified under nerve-winged insects, is very closely related to the Lower-Permian family Archoglossopteridae from the Kuznetsk basin.

*Psocoptera.*—Fossil representatives of psocids have received little attention. It should be noted that Danka (24) started this work when he investigated the extent of individual variability of venation in recent representatives of the order with the view of further, more successful, studies of the fossils. Of some interest are the descriptions of new species belonging to the family Lophioneuridae [Becker-Migdisova (7)] from the Permian deposits of the Kuznetsk basin; these species are closely related to previously known species of North America and Australia.

*Homoptera.*—Of great importance is the finding of two new families of this order in Carboniferous deposits in Belgium and the Kuznetsk basin, i.e., of Protoprosbolidae (72, 73) and Blattoprosbolidae (11), which preserved, along with specific characters of the order, some features of blattoidean ancestors. Becker-Migdisova was completely justified in separating them into a special superfamily, Blattoprosbolidea; she pointed out that they possibly originated from ancient cockroaches of the family Archimylacridae.

In recent years quite a few papers have appeared with descriptions of new species of different geological ages belonging to this order [Becker-Migdisova (2 to 6; 9 to 13); Schmidt (113); Statz (124)]. In each of her papers, Becker-Migdisova discusses the phylogenetic relations of the groups described and their significance in age determination of deposits that include insect remains and draws some palaeogeographical conclusions.

Evans (42, 43) revised all fossil Homoptera and changed their taxonomic arrangement. The reviewer, as well as Becker-Migdisova, a specialist in this group, thinks that Evans' system has an inadequate basis and requires a considerable revision. Thorough investigations of purely Australian ancient faunas are of capital importance since their relation to corresponding faunas of other continents are not very clear.

*Heteroptera.*—The specialists studying recent Heteroptera pay only a little attention to fossil representatives of the order; therefore, only a few papers have been devoted to the description of this group (8, 11, 41, 54, 59, 63, 72, 73, 99, 125, 126).

*Coleoptera.*—Taking into consideration the fact that fossil remains of Coleoptera are represented almost exclusively by their elytra, by which re-

cent beetles are not identified as a rule, the bulk of the descriptions of Cenozoic beetles is relatively unreliable and requires further investigation of the specific characters of the structure of elytra in recent beetles. The same holds true for Mezozoic and Palaeozoic Coleoptera, though it is somewhat easier to study the latter because of their primitive features. The investigation of Permian Coleoptera from the Kuznetsk basin gave an unexpectedly large number of species and an interesting pattern [Rohdendorf (108, 110)] of the alternation of faunistic complexes and of the peculiarities of elytra sculpture in time.

*Neuropteroidea.*—Having described new forms of fossil lacewings from Mesozoic and Palaeozoic faunas of the U.S.S.R., Martynova revised her previously published data on this order and presented a new classification. This classification is far from being perfect; it requires considerable further elaboration since its author does not know all the families of recent Neuroptera and was unable to reinvestigate holotypes of many lacewings from the family Prohemerobiidae of Europe. The most recent Palaeozoic material on this order (93) indicated to Martynova that Neuroptera originated from some branch of Blattoidea and gave rise to the group of orders of the panorpoid complex. The descriptions of new lacewings from the Mesozoic (88, 89, 93, 106, 134) and Cenozoic (1, 98) are of interest.

There has been little study of fossil Raphidioptera; the first snakefly from the Permian of the north of the U.S.S.R. has been described (93), a female with the ovipositor and head of the *Inocellia* type. Very valuable are the descriptions of snakeflies from the Baltic amber [Carpenter (23)], including both genera of the family Inocellidae known at present.

Some species of Megaloptera have been described from the Permian of the U.S.S.R. [Martynova (93)]; Sharov (114) described an alderfly larva, presumably belonging to the same Permian family Permosialidae.

*Mecoptera.*—Most of the material on this group has been reported from the Permian and Triassic of Australia by Riek (101, 102, 104). He presented figures of both new representatives of the species described earlier by Tilliard and of many new forms. It seems that the author does not think it likely to find in the Permian and Triassic of Australia the families and genera previously known from the deposits of other continents of the same age, though a close relationship of fossil forms of the ancient Angaride and of Australian faunas is quite evident. This gave rise to descriptions of many new genera and families, despite the fact that, when judged by the figures, many of them show no differences from previously known taxa. A number of papers have appeared with descriptions of new Tertiary (21, 22) and Mesozoic (84, 85, 90) species of scorpionflies. On the basis of new material from the Palaeozoic of the Kuznetsk basin, where very diverse representatives of the suborder Protomecoptera were found, Martynova has given a phylogenetic assay of the order. In her opinion, the branch Trichoptera-Lepidoptera had separated from one branch of ancient scorpionflies, while Diptera and Hymenoptera had separated from another branch. The phylo-

genetic conclusions of Martynova (92, 94) almost completely coincide with those of Hinton (50), though both authors worked independently on different material. Hinton arrived at his conclusions on the basis of studying anatomy of the larvae of contemporary orders of panorpoid complex, whereas Martynova drew her conclusions on the basis of studying wing venation of fossil scorpionflies.

We do not yet know from which ancient scorpionflies the specialized branch Boreidae arose, but the latter type are true Mecoptera. The anatomy of the larva of such phylogenetic relics (111, 112) like Meropidae, Choristidae, and Nannochoristidae is very likely to differ drastically from the structure of Panorpidae, to the same extent as the structure of Boreidae larva, but nevertheless these are true scorpionflies. Therefore, it is hardly possible to separate them into a special order, just as is the case with Boreidae.

The flourishing of Mecoptera during the Permian and their vigorous speciation, made this order very important stratigraphically (14, 86, 91) since the age of sediments is readily determined by the complexes of genera and species and by the morphological peculiarities of the representatives of the order included within these sediments.

*Trichoptera.*—The finding of two Permian species of caddisflies belonging to the family Microptysmatidae in Eurasia and of a number of species of Australian Cladochoristidae (93, 104) has increased the interest in the study of Mesozoic caddisflies of the family Necrotaulidae. This family should be studied again since it seems that the rise of many tertiary families was evidenced as early as in the Mesozoic.

*Lepidoptera.*—Butterflies are very rarely found as fossils; therefore, a new species of *Cossidae* from the Miocene of the Caucasus, described by Kozhantshikov (55), is very interesting. The figures do not represent it quite correctly; the venation is given an erroneous interpretation. The author once more takes the furcation of the anterior cubitus for its two branches while calling the hind cubitus "the first anal vein," which contradicts the data on the structure of this system in both the fossil and contemporary caddisflies, from which butterflies have originated.

*Diptera.*—In the last decade, there have been descriptions of only Tertiary species of flies (40, 49, 51, 52, 95, 97). All these papers are written by the specialists in recent Diptera and are therefore of high quality. Of particular interest is the paper by Petrunkevitch (97) with the description of a striking species of Ceratopogonidae with the stridulatory organ located on the wings.

*Hymenoptera.*—Almost no papers appeared on this insect group in recent years. Burdick (18) reviewed fossil Xyelidae, which were described earlier. Sharov (115) described the cretaceous family Cretavidae (Aculeata), which seems to have the panorpoid type of the wing venation.

## Stratigraphic Importance of Fossil Insects

Investigations of insects collected in different horizons of continental deposits of the Carboniferous and Permian of the Kuznetsk basin showed

the importance of these collections as indices of geological age. Some insect orders (Glosselytrodea, Coleoptera, Mecoptera) proved to be particularly fine indices. In different strata, they not only have sharply differing complexes of families and genera, but possess also a peculiar morphological specificity of wings in relation to the time of their existence. So, for example, scorpionflies of the Kuznetsk stratum bear on traces of their veins the attachment of large-sized hairs which scorpionflies of higher strata are lacking. The beetles of the Iljinsk-stratum possess a particularly rough structure of the elytra. The width and height of cells of anterior wings in Glosselytrodea alter from stratum to stratum. All these characteristics allow one to draw a conclusion on the age of rocks including fragmentary remains. The age of rocks is determined not more exactly than up to the order. Guthörl (45) shows us the stratigraphical significance of the insects of carboniferous Palaeodictyoptera of Saarbrücken.

*Phylogenetic schemes.*—In his exhaustive review published in *Traité de Zoologie*, Jeannel (53) presents a phylogenetic tree of insects and states that it was worked out by Martynov. But an unfortunate misprint crept into the table, so that the order Strepsiptera is shown to start not from the branch of Coleoptera, as is the case in Martynov's table, but from Hymenoptera. This error seems to be reflected in *Traité de Palèontologie*, since the order Strepsiptera is located here just after the order Hymenoptera. Jan Oberberger even discusses the possibility of accepting "Martynov's suggestion" on the close relationship of Strepsiptera to Hymenoptera.

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# THE ANALYSIS OF INSECT EMBRYOGENESIS[1,2,3]

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## INTRODUCTION

Development has been defined by Seidel (109) as the change of the organism from one structural organization to another. It is the task of the developmental physiologist to describe these organizational states in functional terms, to elucidate the mechanisms through which a specific state is attained, and to determine the relationships that exist between different organizational states.

The causal analysis of insect development has provided some insight into these various aspects of development and will be reviewed here. In general, the discussion will be limited to earlier phases of development and will not be concerned with descriptive aspects of embryology. Because of limitations of space, it has not been possible to include much of the original literature; however, by reference to the papers cited, the present bibliography can be considerably expanded.

Morphological aspects of insect development have been covered in detail by Johannsen & Butt (57) and Hagan (48). Valuable brief discussions are to be found in the texts of Waddington (120) and Weber (122). Most of the techniques utilized in experimental studies are critically discussed in the excellent review by Richards & Miller (92); more recent technical methods are to be found in Waddington (120).

## THE EGG CELL

The insect egg is often bilaterally symmetrical, foreshadowing the eventual orientation of the embryo. It is typically enclosed by a thin translucent vitelline membrane and by an outer, opaque chorion, which is often ornately patterned. There is usually a specialized region in the membranes to allow the entry of sperm, the micropyle. In some species, the egg produces a fertilization cone which extends into the micropyle at the time of sperm entry and which later disappears (4, 27).

The ooplasm consists of yolk globules dispersed in a cytoplasmic network. At the periphery, there is a cytoplasmic border devoid of yolk; this is the cortex or periplasm. The relative proportions of yolk, reticular cytoplasm, and cortex vary from species to species; the variance has been related to the

[1] The survey of literature pertaining to this review was concluded in May, 1960.

[2] I am deeply indebted to my colleagues Dr. W. W. Doane, Dr. R. B. Nicklas, and Professor D. F. Poulson for their constructive criticisms of this review, and to Dr. C. C. Doane for his verification of species names.

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developmental pattern of the species [Krause (65); Seidel (102)]. In general, eggs of hemimetabolous insects have a poorly developed cortex, a paucity of cytoplasm, and an abundance of yolk. The initial embryonic portion of the egg is small compared to the extraembryonic regions and often consists almost entirely of the presumptive procephalic region, the trunk being formed later through the activities of a "segment-forming zone" (64). There is considerable shifting of the embryo within the egg during embryogenesis. Eggs of holometabolous insects, on the other hand, are characterized by a well-developed, often-differentiated cortex, less yolk, and a more extensive cytoplasmic reticulum. The embryonic anlage from the first occupies a proportionately larger region of the egg; extraembryonic regions may be reduced to mere rudiments. The major body regions are represented in approximately the same proportions in the egg as in the fully formed embryo, and the segment-forming zone is of less consequence. There are less dramatic changes in the position of the embryo during development. The major features of development in these different egg types have been diagrammatically represented by Weber (122, Figure 34). While these distinctions between hemimetabolous and holometabolous insects are, in general, valid, there are certain deviations from the scheme (122).

The ooplasmic components show a patterned organization which changes during development (35, 36, 44, 59, 74, 89, 94, 98, 101). There is a gradual separation of cellular elements and cytoplasm from the yolk and an increase in the depth of the cortex, culminating in the establishment of a broad outer layer of cytoplasm, containing the blastoderm nuclei, and a central concentration of yolk and vitellophags.

The dimensions of the entire egg may change considerably during the early stages of development (13, 14, 43, 44, 49, 55). Imiazumi (55) has shown these changes to be inherent in the egg system, for the same pattern of elongation and contraction occurs in developing and in unfertilized eggs. There is a considerable shifting of the cortical layer during early cleavage stages (28); if these cortical movements do not occur, subsequent development may be abnormal (29).

The organization of the egg cell has important consequences for development, and genes that alter egg structure may be important for macro-evolutionary processes. Sterility genes in *Drosophila* provide material for a study of the effects of altered egg structure on subsequent development of the zygote [see discussion in Counce (24)].

## MEIOSIS, FERTILIZATION, AND CLEAVAGE

### General Description

The meiotic divisions occur in a cytoplasmic island associated with the cortex. The four nuclei that result from these two divisions are arranged in linear order at roughly right angles to the long axis of the egg; the innermost of these is the female pronucleus. The fate of the other, or polar body, nuclei varies: all may be extruded (13, 44, 49) or, in other instances, some (47, 61, 76, 94, 111) or all (47, 60) may remain within the egg. Polar body nuclei re-

maining in the egg usually fuse and form a large metaphase plate in the region where maturation has occurred. They evidence no further mitotic activity, and the spindle and chromosomes eventually degenerate (60). Fusion of male and female pronuclei (syngamy) gives rise to the zygote nucleus. With the exception of the Apterygota and some parasitic Hymenoptera, cytoplasmic cleavage and cell membrane formation do not occur until the establishment of the cellular blastoderm (57). The period between syngamy and blastogenesis is generally referred to as "cleavage," but, as Haget (49) has pointed out, the term "intravitelline nuclear multiplication" is more accurate. Nuclear divisions are characteristically synchronous and rapid during this stage (113, 119). Approximately eight divisions occur before nuclei enter the cortex. Possible causal relationships during the period prior to blastoderm formation will now be considered. By the very nature of the egg system, these involve either nuclear or nucleocytoplasmic interactions.

## Sperm

The relation between sperm entry and the initiation of meiosis varies. Fertilization is essential in some types of parthenogenesis (15, 71); however, in most instances, it is not (57). Sperm entry is not required for the completion of meiosis in either *Drosophila melanogaster* Meigen (32) or *Drosophila funebris* (Fabricius) (116). In many orthopterans, development in unfertilized eggs may extend beyond the blastoderm stage (59, 61, 63).

Behavior of the female pronucleus can be influenced by sperm. In unfertilized eggs of both parthenogenetic and bisexual races of the bagworm moth, *Solenobia triquetrella* Fischer von Röslerstamm, the female pronucleus migrates into the anterior cortex; when sperm are present in either egg type, the pronucleus migrates to the center of the egg where syngamy occurs (111). In the mutant *deep orange* of *D. melanogaster*, where there is evidence for an interaction between the pronuclei prior to syngamy (24), the observed developmental effects may be related to the path of migration of the female pronucleus.

It is possible that an interaction between the pronuclei also results in the suppression of division in supernumerary sperm. It has been suggested that suppression is attributable to an effect emanating from the zygote nucleus (119); however, cytological observations indicate that suppression is determined by an earlier event, for supernumerary sperm do not undergo the changes that the male pronucleus exhibits prior to syngamy. In certain abnormal conditions, division of supernumerary sperm is not suppressed, and developmental abnormalities may result (24, 60, 113).

However, more than one sperm may play an active role in the egg. Double fertilization may be responsible for the production of some gynandromorphs (47), and fusion of two spermatozoa to form a diploid synkaryon has been obtained in experimental androgenesis in the silkworms, *Bombyx mori* (Linnaeus) and *Bombyx mandarina* (Moore) (2). The participation of supernumerary sperm in development has been invoked to explain some phenomena in certain female-sterile mutants of *D. melanogaster* (26).

## The Cortex

The cortex plays a significant role in early development. It is assumed that the mitotic block evidenced by polar body nuclei is imposed by their position in the egg cortex (119); therefore, which of the four nuclei resulting from meiosis is to become the female pronucleus is determined by its position in relation to the cortex. In some situations, the block to further division of polar nuclei fails to materialize (47, 60, 111). In parthenogenetic development of *S. triquetrella*, polar body nuclei two and three move out of the cortical region, fuse, and divide to give rise to the embryo. If sperm are present in such eggs, a zygote nucleus results from the fusion of pronuclei. However, there is no mitotic block of the polar body nuclei, and they continue to divide, often resulting in developmental chaos (111). Heritable gynandromorphism in *B. mori* is associated with the tendency for formation of binucleate eggs (47). Significantly, the second nucleus (the fusion product of polar body nuclei two and three) also migrates out of the cortical region if it is to participate in further development. Goldschmidt (46) has proposed a most extraordinary kind of behavior of polar body nuclei in the *Drosophila* mutant *rudimentary* that involves their migration to the posterior pole and their eventual inclusion as germ cells. However, the breeding results for which this interpretation was advanced can probably be explained by more orthodox events. In some instances of automictic parthenogenesis, the cleavage nucleus is derived from fusion of the presumptive female pronucleus and a polar body nucleus (115) or from fusion of the second and third polar body nuclei (71, 77, 111). There is no recorded observation of the most peripheral of the polar body nuclei contributing to parthenogenetic development. Cytological observations of Seiler (111) and Goldschmidt & Katsuki (47) suggest that the developmental fate of polar nuclei is related to their ability to migrate out of the cortical region.

Meiosis is known to be under genetic control (24, 45). The genetic system may act through its effects on the cortex or endoplasm with consequent effects on the mechanics of division; obviously it may also have a more direct effect in some instances.

In many insects, cleavage nuclei have been shown to be totipotent, their developmental fates being determined only some time after their entry into the cortex (74, 80, 88, 105). In determinate eggs, the cortex is assumed to be highly organized, with its specific fate fixed at syngamy; thus, the developmental fate of the nuclei is determined from the moment they enter the cortex. There is evidence, however, that there is a time lapse between the entry of nuclei into the cortex and their final determination and that some nuclei gain the ability for self-differentiation before others. Indeed, there may even be a certain flexibility as far as determination is concerned. These points are illustrated by various kinds of abnormal embryogenesis resulting from ultrasonic irradiation of syncytial blastoderm stages of *D. melanogaster* (30, 31). If early syncytial stages are treated, some embryos show cell proliferation without differentiation, while others form two types of recognizable cells, ganglion cells, and mesoderm cells which may differentiate into isolated

myoblasts (30). When a later syncytial stage is treated, a type of development occurs that is intermediate between these two types, and only ganglion cells are differentiated (31). Since selective destruction of all but presumptive neuroblast cells is hardly a tenable explanation for this, we may assume that these cells acquire the ability of self-differentiation before mesoderm cells. Another indication of a difference in determination between early and late syncytial blastoderm nuclei is that embryos that show abnormal, but organized, development following treatment at the early stage invariably have abnormalities in segmentation (30); comparable embryos derived from treatment at the later stage show normal hypodermal segmentation, although mesodermal tissues may be very abnormal (31). There is also a tendency for presumptive hypodermal cells to differentiate into ganglion cells, an indication that mosaicism is not as fixed as has been suggested (30).

It should be emphasized that there is obviously an important change in the nature of the cortex between the maturation divisions and blastoderm formation, for, without exception, the effect of the cortex is deleterious to nuclei [polar body nuclei (see above), female pronuclei and their descendents (111), or sperm (72)] that enter or remain in it in early developmental stages. Only later does it provide an environment that is conducive to normal division and development.

### The Endoplasm

The non-cortical cytoplasm also plays a part in developmental events. The endoplasm acts as the controlling factor in insects in which chromosome elimination occurs as a normal feature of embryogenesis. In the gall midges (51, 78), several chromosomes are eliminated from all but the germ line nuclei during early cleavage; experimental evidence (78) indicates it is the characteristics of the cytoplasm surrounding nuclei that determine whether they are to be somatic or germinal.[4] Chromosome elimination is also known in the diaspine scale, *Pseudaulacaspis pentagona* (Targioni) (15), and the armored scale, *Aspidiotus simulans* De Lotto (16). All early embryos are diploid, but paternal chromosomes are eliminated in male embryos during late cleavage. Males are produced by aging or by x-rayed females; it has been suggested that the physiology of the mother may condition the ooplasm in such a way that elimination is controlled or induced.

In certain sterility mutants of *D. melanogaster*, zygotic nuclei (or their descendents) with identical genotypes suffer very different developmental fates in different ooplasms (24). Detrimental interactions between nucleus and cytoplasm in certain *Drosophila* hybrids (60) and interracial crosses in mosquitoes (71) result in embryonic death. A similar interaction has been proposed to explain the general debility observed in heterospermic androgenesis in *Bombyx* (2). Irradiation of cytoplasm without damage to nuclei is sufficient to cause death or abnormal embryogenesis (118, 119) in Diptera and Hymenoptera.

[4] See also Geyer-Duszyńska, I., "Experimental Research on Chromosome Elimination in Cecidomyidae (Diptera)," *J. Exptl. Zool.*, **141**, 391–447 (1959).

### Cleavage VI

It has been suggested, on the basis of experimental evidence, that there are important and fundamental changes occurring in the insect egg at or about Cleavage VI (119). Indeed, this does represent a critical or sensitive phase in many different species, although all the changes observed are not necessarily related to the same cause. In many species, migration of nuclei toward the periphery begins at this period [e.g., in the Mediterranean flour moth, *Anagasta kühniella* (Zeller) (101)] and is often accompanied by loss of synchrony (13, 34, 94). In the yellow mealworm, *Tenebrio molitor* Linnaeus (44), Cleavage VI marks the separation of future embryonic and extraembryonic cells by their position in the egg, and there is a transition from mosaic to regulative development. In the alder fly, *Sialis lutaria* (Linnaeus), this division marks the transition from regulative to mosaic development; the cortex is established at this same division (35, 36). In certain embryonic lethals in *Drosophila*, development never proceeds beyond this division (33, 60). It may also mark the end of cortical movements during cleavage in *Drosophila* (28). In some scale insects, the polar body nuclei, which have remained quiescent until Cleavage V, unite with cleavage nuclei, divide, and give rise to the polyploid cells of the symbiont organ. At the next division (VI), chromosome elimination begins in the diploid cells of male embryos (15, 16). Thus, some significant developmental change at this period has been observed in Hemiptera (94), Coleoptera (44), Neuroptera (35, 36), Lepidoptera (101), and Diptera (13, 33, 34, 60, 119). What the underlying mechanisms are, remains to be demonstrated.

## POLE CELLS

In some insects, primordial germ cells are differentiated from somatic cells before or at the time of blastoderm formation (102, 113); in others, they become apparent only at a relatively late stage in development and are attached to the walls of the coelom (102). However, as Seidel (102) points out, in the latter instance, germ cells must in some way be differentiated from somatic cells at a much earlier stage in development, for they alone do not respond to the activities of the Differentiation Center (see below) but develop according to their own pattern.

There has been considerable interest in the developmental fate of the pole cells in *D. melanogaster* (87). They re-enter the embryo at two different stages: after formation of the cellular blastoderm (Phase I) and during extension of the germ band when they are transported by the developing posterior mid-gut anlage (Phase II) (86, 113). Pole cells that migrate at Phase I were at first believed to give rise to vitellophags, the germinal elements of the gonad being derived from pole cells that migrate during Phase II. However, morphological observations suggested to Poulson (86) that pole cells that migrate at Phase I give rise to primordial germ cells and that those entering during Phase II give rise to specialized portions of the mid-gut. Gonad formation in an embryonic lethal in which Phase II migration is never accomplished proves that pole cells migrating at Phase I can give rise to germ cells

(29). Experimental data obtained by Poulson & Waterhouse (87) also support the conclusion that pole cells migrating at Phase I are essentially gonadal and those migrating at Phase II are involved in mid-gut formation. However, Hathaway [quoted in Waddington (121)], on the basis of results in similar experiments, concludes that germ cells are derived from pole cells that migrate at Phase II. Observations on other *Drosophila* species suggest that migration is not genuinely diphasic but continuous [Counce (unpublished)]; if this is also true in *D. melanogaster*, it may, in part, account for the apparently contradictory results of Hathaway and Poulson & Waterhouse.

## BLASTODERM FORMATION

The possible mechanisms involved in migration of nuclei to the periphery to form the blastoderm are discussed by Richards & Miller (92). Changes in nuclear position associated with division are not sufficient to give rise to a typical blastoderm (30, 84, 114). It is essential that the integrity of the cortex be maintained; thus, abnormal development of the cortex produces subsequent abnormalities in nuclear distribution during blastogenesis (12, 35, 36, 41, 42, 44, 90, 91, 95, 96). When nuclei fail to enter the cortex, nuclei from adjacent areas do not move into these gaps and there is localized cortical breakdown (24, 84). Cytoplasmic cleavage in the absence of nuclei has been demonstrated only in the moth *Phragmatobia fulginosa* (Linnaeus) (110).

## FORMATION OF THE GERM ANLAGE

In the insect, only a part of the blastoderm is destined to give rise to the embryo. When it is first differentiated from the regions that will give rise to extraembryonic membranes, it is spoken of as the "embryonic" or "germ anlage" (=primordium), as distinct from the "germ band," which represents the stage in development when the primary germ layers are established. The germ anlage may be formed by differentiation *in situ* or by cell migration [see Weber (122)]. There is considerable variation in size and structure of the germ anlage in the various insect groups (63, 65, 122).

## THE YOLK SYSTEM

The yolk system consists of the deutoplasm, the reticular cytoplasm that does not contribute to formation of the cellular blastoderm, and the yolk nuclei or vitellophags. The yolk system serves not only as a source of nutrients for the developing embryo, but is also a significant morphogenetic agent (106).

In all the orders that have been carefully studied, localized retractions of the yolk system, produced either by liquifaction or contraction, are associated with form changes of considerable magnitude (3, 75). Morphogenetic movements of the yolk system are involved in formation of the germ anlage (23, 44, 63, 67, 73, 106), establishment of the germ band (43, 75, 94, 98, 123), pole cell formation (94), blastokinesis (3, 64, 70, 73, 95), and mycetome movement (3, 94). The presence of yolk is required for closure of the ventral

furrow (but not its formation) in the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), and continued contact with the yolk is essential for normal morphogenesis (49).

Developmental changes in the yolk system are largely independent of the progress of development in the embryo (44, 73, 75). Yolk cleavage, but not localized contraction, can occur in the absence of the germ anlage (44, 70, 73, 106). Coagulated yolk within the system does not prevent yolk cleavage, but an unhealthy or degenerating germ anlage does (70).

The viscosity of the yolk increases during development (44, 74, 75, 99, 106). Its chemical properties may also change (54, 64, 75). Dramatic changes in the optical properties of the yolk precede formation of the germ anlage in the damselfly, *Platycnemis pennipes* (Pallas) (106); similar, but less pronounced, changes also occur in the stone cricket, *Tachycines asynamorus* (Adelung) (67), and *Notonecta* (68).

A yolk sac or primitive gut forms beneath the blastoderm; it is a syncytial structure composed of vitellophag nuclei and peripheral cytoplasm (3, 94). The yolk sac plays a role in yolk cleavage and in the movement of certain structures associated with symbiotic organisms (3). It may act as a mold in gut formation and may also contribute cells to the mid-gut epithelium (14, 54, 56, 75, 86). Contraction of the sac appears to draw yolk into the mid-gut as it nears completion (43).

The effective control of many of the activities of the yolk system is undoubtedly exercised by the vitellophags. Müller (75) has studied the developmental history of vitellophags in the honey bee, *Apis mellifera* Linnaeus. Three groups of vitellophags (primary, secondary, and tertiary), differing in time and place of origin and in function, were identified. The major function of primary vitellophags is to increase plasmatic structures during cleavage through assimilative alteration of yolk. Secondary vitellophags contribute to the production of new cytoplasm but also cause localized liquefaction of yolk, an essential step prior to yolk contraction. Tertiary vitellophags are responsible for the actual contraction of the yolk and for breakdown and utilization of yolk in the mid-gut; they may also form part of the mid-gut epithelium.

## THE DYNAMICS OF DEVELOPMENT

As Seidel has repeatedly emphasized (108, 109), development is characterized by a co-ordinated series of events in which there is an alternation between morphogenetic movements and activation of physiological centers. Although Seidel was not the first to utilize experimental techniques in the study of insect development, his were the first concerted attempts to elucidate the mechanics of insect embryogenesis. These studies have been reviewed in detail elsewhere (6, 7, 57, 67, 68, 69, 83, 92, 105, 107, 108, 120, 122). The interrelations of developmental systems have not been completely worked out in any one insect; therefore, the following scheme is a composite one.

## Morphogenetic Movements

Morphogenetic movements are those diverse movements of cells and groups of cells leading to changes in form; they are an integral part of development and differentiation. Through morphogenetic movements, new relationships arise, permitting new combinations and reactions to take place, which, in turn, give rise to new potentials for development. In insects, as in other forms, we know little of the forces involved in these movements [discussion in Waddington (120, p. 415 ff.)].

Among the large-scale morphogenetic movements in the insect embryo are yolk contraction, gastrulation, blastokinesis, extension and contraction of the germ band, dorsal closure, and condensation of head segments (19). In cyclorraphous Diptera, the characteristic acephalic larval condition is the consequence of involution of the head segments and extension of the thoracic segments during embryogenesis (17, 43, 86).

The importance and variety of morphogenetic movements of the yolk have been discussed in the previous section. In *Drosophila*, gastrulation appears to be essential for normal differentiation (41, 42, 84, 85), as is blastokinesis in *Tachycines* (69) and *Platycnemis* (104). Head involution in *D. melanogaster* is a critical phase in development and is easily disturbed by slight deviations in embryogenesis (17, 25, 37). However, many other morphogenetic movements, such as dorsal closure and shortening of the germ band, evidence considerable independence (5, 38, 49, 68, 97, 104).

Experimental studies indicate that expansion and spreading movements of ectoderm are involved in many morphogenetic movements and that association with mesoderm is essential for the occurrence of such formative movements (5, 49, 67).

## Control Centers

*The Cleavage Center.*—The Cleavage Center [*Furchungszentrum* (63)] is the first identifiable center of control. In this region, cleavage of the zygote nucleus occurs, and primary vitellophags are first differentiated; at this same level in the egg, migration of secondary vitellophags from the blastoderm begins, and the anterior edges of the germ are delimited (67). It seems probable that activation of the Cleavage Center is associated with sperm entry. In *Tachycines* the Cleavage Center is not essential after nuclei have reached the posterior half of the egg, but in *Notonecta* it is required up to the time of formation of the differentiated blastoderm (68).

*The Activation Center.*—This was the first of the control centers to be experimentally demonstrated [*Bildungszentrum* (103, 105)]. In *Platycnemis*, the Activation Center, located in the posterior polar region, is activated by the entry of cleavage nuclei into the polar region. Activation of the Center is a prerequisite for the formation of a germ anlage. The first apparent effect of activation is a change in the optical properties of the yolk as some "agent" diffuses from the posterior toward the anterior. Anterior regions are activated by the diffusion of the agent and become independent of regions pos-

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terior to them. Once the germ anlage has been established, the Activation Center is no longer required. The Center acts by effecting a change in the yolk, which is essential for the occurrence of localized yolk contraction under stimulus of a third center (see below); yolk cleavage is apparently independent of the Center (105).

Other investigators have attempted to demonstrate the existence of comparable Activation Centers in other species (12, 44, 68, 73, 89, 90, 91). The "demonstration" of such Centers however has not been completely convincing in most instances [for a critical evaluation see Haget (49)]. Nevertheless, the results of these experiments and the general sensitivity of early stages to any experimental intervention suggest that this stage has some developmental significance not yet fully understood and that the region of the posterior pole is involved in some special way.

The experimental studies of Sander (95, 96) on the cicada, *Euscelis plebejea* (Fallén), demonstrated that a factoral region at the posterior pole is of major consequence in the development of this insect; he equates this region to the Activation Center in *Platycnemis*. Displacement of polar cytoplasm can be accomplished by depression of the pole with a blunt needle; a ball of symbionts at the posterior pole provides a convenient marker for the extent of displacement. In an extensive series of experiments involving displacement, constriction, and displacement plus constriction, Sander demonstrated the existence of anterior and posterior "formative factors," which probably act in opposing gradients. These factors are responsible for determining the exact length of the germ anlage and the formation and site of differentiation of segments. Anterior formative factors are essential for formation of the head segments, and posterior factors are unable to express themselves where anterior factors are at a maximum concentration.

*The Differentiation Center.*—The next Center to be activated is the Differentiation Center [*Differenzierungszentrum* (102, 106)]. A Differentiation Center has been demonstrated in almost every insect embryo investigated and is invariably associated with the presumptive prothorax region (49). It acts as a control center for developmental events over a considerable span of time. Its activity may first become apparent at the time of cleavage (36) or at the time of migration of nuclei toward the periphery (14), or as late as the appearance of external segmentation (44, 101). In some forms, it is differentiated morphologically from the rest of the blastoderm (98) or shows biochemical differentiation (8, 66, 124). It is the last of the control centers to act while the germ anlage still represents a single functional unit (67).

The Differentiation Center controls formation of the germ anlage or germ band, or both, in many insects. In *Platycnemis* (106), this is accomplished through a change in the division rate of blastoderm nuclei in time and space and by a localized contraction of yolk beginning in the region of the Center and progressing anteriorly and posteriorly to it. The space created by yolk contraction acts as a mold for formation of the germ anlage, and a second anlage can be produced by causing yolk contraction elsewhere (106). Mesoderm formation usually begins in this region (98); when it begins else-

where, closure of the ectoderm beneath the mesoderm frequently begins at the Differentiation Center (94). Segmentation and organogenesis are first apparent in the thoracic region (4, 54, 59, 67).

In *Tachycines*, the Differentiation Center determines the anterior-posterior polarity of the germ anlage and the invagination tendency, along the ventral mid-line, which is involved in the origin of mesoderm. The establishment of the major body regions—procephalon, gnathocephalon, thorax, and abdomen—is controlled by the Differentiation Center (67, 70).

The Differentiation Center may shift toward the anterior in later phases of embryogenesis (10, 36, 67, 98). Some developmental events, such as differentiation of germ cells (102) and dorsal closure, are apparently not under control of the Center.

*Segment Centers.*—With closure of the ventral furrow, new centers become active within each of the major body regions; these centers are associated with the establishment of segmentation and of bilaterality. Krause (67) terms them "second-order Differentiation Centers" or Segment Centers. They are first established in the mid-ventral region of each segment, in sequence, both anteriorly and posteriorly to the Differentiation Center (4, 67). The effects of the Segment Centers radiate toward the sides of the germ band and lead to the establishment of bilaterality. The Centers, in all probability, work through the co-ordinated action of the germ layers. In *Tachycines* for example, segmentation does not occur in the absence of mesoderm (67). The position of the mesoderm determines the ventral mid-line; then, through the process of intradermal induction and its subsequent effects on mesodermal differentiation, bilaterality is established. Differentiation occurs in the same sequence in every segment; the rate at which it occurs is specific for each segment, the total differentiation process in posterior segments requiring considerably less time than in more anterior ones (4). The failure of regulation if healing occurs between different segments after the germ anlage is split suggests that these Centers are very specific in nature (70).

## Reaction Systems

Four kinds of reaction systems have been suggested by Krause (68). The *ooplasmic reaction system* consists of the entire egg during cleavage and later is confined to the endoplasm; with the occurrence of yolk cleavage, it is disrupted as a system. (It should be pointed out that, even during early stages of development, there are, in all probability, at least two systems present in the egg: a cortical system and an endoplasmic system; for all evidence points to a fundamental and important difference between the cortex and the rest of the cytoplasm.) At blastoderm formation, new systems are established: *intrablastemic reaction systems* composed of uniform cell complexes and *interblastemic reaction systems* composed of two such complexes. At later stages, the possibility for *organ-organ reaction systems* exists.

*Ooplasmic reaction systems.*—Several examples have been given in the discussion of physiological centers. The reaction chain leading to the activation of the Differentiation Center and formation of the germ anlage in

*Platycnemis* involves reactions within the ooplasmic system. The later morphogenetic activities of the yolk system are examples of ooplasmic reaction systems limited to the endoplasm.

*Intrablastemic reaction systems.*—The most striking example of such a system in the insect embryo is the ectoderm layer. In insects, the ectoderm is a self-differentiating system [Bock (5); Haget (49)]. This ability is gradually acquired by a process of "intradermal induction" (49) beginning from the region of the Differentiation Center and extending both anteriorly and posteriorly from this point; toward the end of gastrulation, the ability for self-differentiation will have been acquired by the entire embryonic ectoderm (49). Within each segment, the ability for self-differentiation extends from the ventral region toward the sides (4, 5, 49).

*Interblastemic reaction systems.*—Among these, we include all inductive systems. The first inductive system in insects was demonstrated experimentally by Bock (5) in the golden eye, *Chrysopa perla* (Linnaeus). Ectoderm and mesoderm constitute an inductive system, in which the ectoderm is the inductor. Cells of mesoderm are totipotent, their differentiation being determined by their position with respect to the ectoderm. The spreading movements of the mesoderm following gastrulation, however, are independent. Growth and differentiation of ectoderm are not affected by the absence of mesoderm, but formative movements may be. Similar inductive relationships have been experimentally demonstrated in *Leptinotarsa* (49), *Platycnemis* (108), and *Tachycines* (70). In *Tachycines*, the mesoderm has a more direct effect on events in the ectoderm than it has in *Chrysopa* (67,108).

It is probable that a similar inductive relationship exists in *D. melanogaster*. The *Notch* embryo is characterized by hypertrophy of the nervous system, incomplete hypoderm formation, and almost complete failure of mesodermal differentiation. Myoblasts are found only in regions where hypoderm is present [Poulson (85)]. These observations suggest that intradermal induction is abnormal in *Notch* embryos, with consequent disturbances in interblastemic induction. This interpretation is strengthened by the observation that there is a direct correlation between development of hypoderm and the extent and position of myoblast differentiation [Poulson (personal communication)]. The mesoderm spreads out just as it would in normal development; this is identical to the situation in the experimental animals of Bock and of Haget. The mutant factors *×20* (40) and *vestigial*$^{B}$ (18) also seem to affect the process of intradermal induction. It appears that only the relatively undifferentiated hypoderm is an effective inductor, for in none of these mutants does the mesoderm respond to differentiated nervous tissue. These mutants illustrate the valuable inferences that may be drawn in regard to the role of genes in developmental systems about which other information has been derived experimentally. Without the evidence of Bock and of Haget on the nature of intradermal induction, it would not have been possible to interpret so exactly these findings in *D. melanogaster;* on the other hand, these mutants provide us with a key to an understanding of the nuclear control of developmental systems.

An inductive relationship between visceral musculature (inductor) and mid-gut has been demonstrated in *Leptinotarsa* (49). Developmental relationships, which may also be related to induction, have been shown to exist between the eye and optic lobes (82), stomodaeum and anterior mid-gut (97), and wing and wing muscles and ocelli (62). Although the mesodermal elements of the gonad are apparently capable of self-differentiation (1, 50, 121), germ cells in *D. melanogaster* can induce sheath formation by other mesodermal cells when they end up in atypical positions (30, 31). Germ cells of *Leptinotarsa*, however, lack this ability (50). A complex and unexplained situation is presented in hymenopterans by asexual larvae in polyembryonic broods that lack not only gonads but whole somatic systems. It has been postulated that germ cells possess some organizing potential for these systems (112).

*Organ-organ reaction systems.*—Jones (58) has demonstrated that "late embryogenesis from the time of the moult onwards is controlled by an organized endocrine system" in the locusts, *Locustana pardalina* Walker and *Locusta migratoria* (Linnaeus). Pflugfelder (81) has shown a difference in the developmental response of different embryonic tissues of the stick insect, *Carausius morosus* Brunner, transplanted into hosts in which the hormone balance is abnormal.

## Regulation and Determination

The ability of an egg to readjust itself toward normal development following interference varies from species to species. At one end of the spectrum are forms in which a considerable amount of regulation can occur even fairly late in development (indeterminate or regulative eggs) and at the other, forms in which even slight defects at early stages are not repaired (determinate or mosaic eggs). As development progresses, the ability of indeterminate eggs to regulate declines, and development eventually becomes determinate; the two ends of the spectrum therefore are simply manifestations of a time differential in the determination process. Moreover, there is an alternation throughout development between periods of determination and regulation in various systems. The above distinction is based solely on the response of the egg to interference at early developmental stages.

Seidel (102, 107), on the basis of egg structure in different orders, proposed a series of developmental types ranging from indeterminate to determinate egg types. Classified as indeterminate were the Odonata, Hemiptera, and Orthoptera; incompletely determinate were Coleoptera, Hymenoptera, and Lepidoptera; and determinate, Diptera. The validity of this scheme and exceptions to it are discussed elsewhere (92, 122). It is however apparent that whether one obtains evidence for regulation may depend upon the stages of development considered, techniques employed, and, finally, what one considers regulation to be.

This point is well-illustrated by results obtained in the Coleoptera. On the basis of early experiments, it appeared that development in *L. decemlineata* was completely determinate (49, 92). Using new and quite ingenious

techniques, Haget (49) demonstrated that there is regulation at early stages. In *T. molitor*, the egg responds as a mosaic type until Cleavage VI; after this time, it demonstrates an increasing ability for regulation, which reaches a peak only at the time the germ anlage is established [Ewest (44)]. Brauer (8, 9, 11) succeeded in obtaining duplications and twinning in *Callosobruchus maculatus* (Fabricius), indicating a regulative ability quite equal to that of so-called indeterminate types.

Duplication is considered evidence of regulation. It may arise through the splitting of a single anlage or through incomplete fusion of two (22, 23, 72); complete duplication results in twinning. Duplications have been observed in the following forms (* indicates twinning): Odonata: *Platycnemis** (104); Orthoptera: *Tachycines** (66, 70), *Gryllus* sp. (59), phasmids* (20 to 23), and *Melanoplus differentialis* (Thomas) [R. Jaqua (personal communication)]; Hemiptera: *Euscelis* (96); Coleoptera: *Leptinotarsa* (49) and *Callosobruchus** (8, 9); Neuroptera: *Sialis* (36); and Lepidoptera: *Tineola*?* (72) and *Anagasta* (74).

Duplication and twinning in phasmids deserve some comment. They arise, not from a single anlage, but as a consequence of the formation of compound eggs by fusion of oocytes. Depending upon the component parts and their orientation, compound eggs may give rise to giant embryos formed by fusion of two germ areas or to twins, which from the first develop as a separate anlage within the same egg. Between these extremes are embryos showing varying degrees of duplication depending upon the pattern of egg fusion. Experiments of Pflugfelder (83) suggest, however, that duplication of a single anlage can be obtained in simple eggs of phasmids.

Although no twinning has been obtained experimentally in the Hymenoptera, the variable numbers of embryos that develop from a single egg in polyembryonic species indicate a considerable plasticity in development (112).

Regulation after reduction of egg volume by constriction or after removal of part of the germ anlage may give rise to a single embryo. This may be a harmonic dwarf embryo, but often there is some distortion in its proportions (104). When the part that is removed does not contribute directly to formation of the embryo, regulation may not involve the germ anlage materially; however, there is regulation in the sense that normal developmental processes occur in a restricted space. Regulation after reduction has been observed in Odonata (104), Orthoptera (68), Hemiptera (95), Coleoptera (44), Hymenoptera (89, 100), Neuroptera (36), and Lepidoptera (74).

It has been generally accepted that the dipteran egg is mosaic in nature, at least from the moment of fertilization (52, 53). Experimental data in support of this thesis, however, are not entirely convincing (52, 53, 80, 88). For example, although only 16 per cent of the adults showed abnormal development following removal of protoplasm from early egg stages (52), this was considered evidence for mosaic development! Abnormalities in differentiation observed after ultrasonic treatment suggest that the fate of cells, especially those of the ectodermal layer, is not as irrevocably fixed as would be

expected in mosaic development (30, 31). Nitschmann (79) has interpreted the results of constriction experiments in *Calliphora* sp. as indicative of regulation; he also points out that this explanation fits some of the results obtained by Pauli (80).[5]

Dipteran embryos may also show false regulation or compensation. When some of the presumptive mid-gut cells fail to participate in gut formation, the yolk is enclosed by the remaining endoderm cells. This is accomplished, not through an increase in cell number or cell size, but by a change in cell morphology, resulting in a gut that is complete but cytologically abnormal (25, 29, 38, 39, 87, 88).

When the component parts of a reaction system are capable of shifting in time and space to meet altered developmental conditions, regulation can occur (70, 106). Prior to establishment of the germ anlage, this may involve the entire egg system (99, 100). Once the germ anlage is established, regulation is accomplished by a reorganization within the anlage controlled by the Differentiation Center (67, 70). Regulatory events may also occur within the major body regions (70, 99) or within individual segments (70).

In *Apis* (99, 100), it has been demonstrated that regulation is associated with a shift in the position of the visible or "morphological" Differentiation Center; if its position is not altered, subsequent development is mosaic.

With closure of the ventral furrow, Segmental Centers become the active agents in regulation (70). Regulation is not accomplished by a harmonious change within the whole system ("dynamic regulation") but by "inductive regulation" or assimilation, acting through the intradermal induction system. At early stages, the entire segment regulates if edges of the same segment heal together; with the spread of symmetrization and the ability for self-differentiation, regulation is limited to more and more restricted areas (70). The histological events occurring during regulation have been described in *Apis* (97) and *Tachycines* (70).

The process of regulation through the reorganizational activities of physiological centers is typical of the field systems in development [see discussion in Waddington (121)]. It results in the re-establishment of organization that will permit a part to give rise to an organized whole or in reorganization after fusion of two such centers to produce a single co-ordinated system.

## Conclusions

The causal analysis of insect development has demonstrated the complexity of events involved in development and has provided some insight into the mechanisms by which these events give rise to new structural organization. The chain of events prior to gastrulation, which leads to the establishment of an organized embryo, is characterized more completely in insects than in any other group of organisms. The existence in the insect embryo of developmental phenomena such as field action, induction, and nuclear-cytoplasmic interaction have all been demonstrated experimentally.

[5] See also Idris, B. E. M., "Die Entwicklung im geschnürter Ei von *Culex pipiens* (L.) (Diptera)," *Wilhelm Roux' Arch. Entwicklungsmech. Organ.*, **152**, 230–62 (1960).

To date, development has been carefully analyzed in only a few insects. New techniques, such as those developed by Haget (49), Krause (68), Poulson (85), and Sander (96), should be utilized, not only in the study of additional species, but to re-examine embryogenesis in forms that have been studied by less satisfactory methods.

Obviously many problems remain unsolved. What differences, if any, exist between fine structure organization in regulative and mosaic eggs? What changes occur in regulative eggs in the process of determination? In spite of the fact that the action of specific centers is predictable in time and space and often accompanied by obvious physical changes, no systematic attempt has been made to characterize, in more precise physical or chemical terms, the nature of these centers or the mechanisms by which they act. The fascinating problems hinted at, but unresolved, in the development of phasmids (20 to 23) and polyembryonic forms (112) deserve experimental elucidation. Although important morphological variations are produced by a variety of environmental stimuli acting during embryogenesis (93, 117), only in the aphids has the embryonic mechanism responsible for this developmental plasticity been investigated (62). These are only a few of the problems that challenge the insect embryologist.

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# THE CHEMISTRY OF ORGANIC INSECTICIDES[1,2]

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The intense effort devoted to the development of new chemicals for insect control during the past decade has led to the discovery of a vast number of toxic compounds of widely varying structures. Probably the largest concentration of effort in recent years has been with the organophosphorus compounds, as indicated by the three dozen or more of these compounds in commercial or semicommercial use as of 1958 (61). More recently, largely through the successful usage of Sevin® (1-naphthyl N-methylcarbamate) as a wide spectrum insecticide, esters of carbamic acid have also been examined as potential insecticides. For the most part, this review will be concerned with the two classes of compounds mentioned above, with particular emphasis given to the relationship between chemical structure and insecticidal activity.

The problems of widespread development of resistance by insects to the chlorinated hydrocarbon insecticides (13, 14, 60) and increasing evidence of the presence of significant residues of unchanged chlorinated hydrocarbons in various food products, especially in milk (39, 57, 70, 83), have contributed significantly to the decrease in interest in the development of new compounds of this type. However, a few new organochlorine compounds with high insecticidal activity have been discovered recently, and these will be mentioned briefly. The chemistry, toxicology, and mode of action of these compounds have been reviewed extensively (45, 58, 60, 77, 81).

The chemistry and mode of action of the organophosphorus insecticides have been reviewed in several articles (15, 29, 60, 80). It is apparent that these compounds exert their toxic action through the inhibition of the cholinesterase enzymes. *In vitro* measurements of cholinesterase inhibition and its correlation with insecticidal toxicity and chemical structure have been useful in guiding chemists in the synthesis of new active compounds. It is the intention of this review to discuss some of the more recent thinking among chemists in their quest for new insecticides. In his search for new compounds, the chemist must keep in mind a number of objectives. For obvious reasons, he will seek out compounds that are high in insect and low in mammalian toxicity. For economic reasons, he must consider the cost of manufacturing the new insecticide. The chemist, co-operating with the entomologist, sometimes must direct his program to the synthesis of an insecticide specific for a particular situation. In addition to these and other objectives, the chemist

[1] The survey of literature pertaining to this review was concluded in April, 1960.

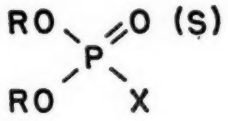
[2] The common names and abbreviations for insecticides in this chapter follow the usage of the Committee on Insecticide Terminology of the Entomological Society of America, as presented in the *J. Econ. Entomol.*, **52,** 361–62, 1032 (1959).

must consider the possibility of insects becoming resistant to the chemicals that successfully control them today. Thus, the bench chemist will continue to synthesize new and different types of compounds, seeking chemicals specific to destructive and harmful insects and always keeping in mind the ever-present spectre of the problem of insecticide resistance.

## Phosphonic and Phosphinic Acid Esters

Of the many thousands of chemicals prepared and evaluated as insecticides in recent years, probably the largest proportion of those tested belong to the organophosphorus family. The majority of these compounds have been derivatives of phosphoric acid, i.e., esters and amides of phosphoric, phosphorothioic, or phosphorodithioic acids. The two outstanding compounds, other than derivatives of phosphoric acid, now in commercial use are Dipterex® (*O,O*-dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate) and EPN (*O*-ethyl *O*-*p*-nitrophenyl phenylphosphonothionate). Of these, Dipterex may be regarded as a derivative of phosphoric acid, since its toxic action is probably exerted through DDVP (2,2-dichlorovinyl dimethyl phosphate), a compound formed spontaneously from Dipterex at pH 6 or higher (9, 62, 66). Thus, EPN, an ester of phenylphosphonic acid, remains the sole non-phosphoric acid derivative among the organophosphorus insecticides.

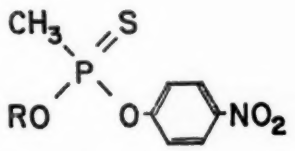
Pesticide manufacturers have concentrated on phosphoric acid esters for the simple reason that they are somewhat easier to synthesize, hence, less costly to manufacture. Thus, the basic structure of a typical organophosphorus insecticide may be shown by the structure below, where R is either methyl, ethyl, or isopropyl and X is a group that is readily susceptible to displacement by a nucleophilic agent. We are limited to R equalling methyl,



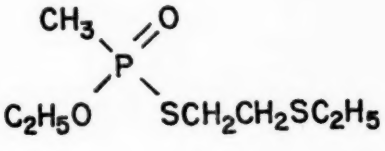

ethyl, or isopropyl, since increasing the alkyl chain leaves the molecule void of insecticidal activity (64, 85, 86). The manufacturer, then, must vary X in his search for newer and better insecticides, and this has been done with great success.

Schrader (78), as early as 1947, described several *O*-ethyl *O*-substituted phenyl methylphosphonates and gave fragmentary information as to their insecticidal activity. More recently, several Russian articles have appeared in which the insecticidal potency of esters of alkylphosphonic and dialkylphosphinic acids has been described. Paĭken *et al.* (74), in a conference in 1955, described the contact toxicity of methylphosphonate analogues of

parathion and demeton against *Pseudococcus* and *Eurygaster* and found them to be as toxic to these insects as their phosphoric acid counterparts. Razumov *et al.* (75, 76), at the same conference, described the preparation and properties of a large series of biologically active alkylated amide esters and mixed esters of alkylphosphonic acids. Among the alkylated amides, the most active cholinesterase inhibitor was ethyl *N*-ethyl ethylphosphonoamidate. This compound was reported to inhibit cholinesterase at $1\times10^{-6}$ *M* and is most



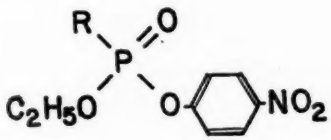
Phosphonate analogue of parathion ($R=CH_3$ or $C_2H_5$)



Phosphonate analogue of demeton

surprising, since the ethylamido moiety is not easily displaced from the phosphorus atom. Among the alkyl ethylphosphonic acid esters of *p*-nitrophenol, the most potent cholinesterase inhibitor was ethyl *p*-nitrophenyl ethylphosphonate, which showed anticholinesterase activity at $1\times10^{-9}$ *M*. This compound, called armin, is used clinically in Russia for the treatment of glaucoma.

Fukuto & Metcalf (30) independently investigated the effect of the alkyl group R on the general reactivity and insect toxicity of a series of ethyl *p*-nitrophenyl alkylphosphonates of the general structure shown below. It was found that the rate of alkaline hydrolysis of these alkylphosphonates to *p*-nitrophenol and ethyl alkylphosphonic acid generally decreased with increasing chain length. Branching, particularly in the 1 and 2 carbon atoms of the alkyl chain, greatly decreased the hydrolysis rate. The first-order hydrolysis



constants ($K_{hyd.}$) were, in general, parallel to the second-order fly-brain cholinesterase inhibition constants ($K_e$). The rate constants, $K_{hyd.}$ and $K_e$, and fly toxicity data are given in Table I. Close examination of the data brings

TABLE I

RATE CONSTANTS AND TOXICITY DATA FOR ETHYL *p*-NITROPHENYL ALKYLPHOSPHONATES*

| | R | $K_e$ (1/moles/min.) | $K_{hyd.}$ (min.$^{-1}$) | $LD_{50}$ female house fly (μg. per gm. fly) |
|---|---|---|---|---|
| I | $CH_3$ | $7.6\times10^{6}$ | $2.42\times10^{-3}$ | 1.0 |
| II | $C_2H_5$ | $1.5\times10^{7}$ | $5.06\times10^{-4}$ | 1.2 |
| III | *n*-$C_3H_7$ | $1.0\times10^{7}$ | $4.17\times10^{-4}$ | 2.0 |
| IV | *n*-$C_4H_9$ | $4.0\times10^{6}$ | $4.23\times10^{-4}$ | 2.4 |
| V | *n*-$C_5H_{11}$ | $8.9\times10^{5}$ | $3.62\times10^{-4}$ | 12 |
| VI | *n*-$C_6H_{13}$ | $6.9\times10^{5}$ | $3.56\times10^{-4}$ | 62 |
| VII | *i*-$C_3H_7$ | $1.1\times10^{5}$ | $1.07\times10^{-4}$ | 28 |
| VIII | *i*-$C_4H_9$ | $3.9\times10^{5}$ | $2.34\times10^{-4}$ | 9 |
| IX | *i*-$C_5H_{11}$ | $4.9\times10^{6}$ | $2.45\times10^{-4}$ | 7 |
| X | *i*-$C_6H_{13}$ | $2.0\times10^{6}$ | $3.62\times10^{-4}$ | 40 |
| XI | *t*-$C_4H_9$ | 6.7 | $3.2\times10^{-6}$† | >5000 |
| XII | 4,4-Dimethylpentyl | $6.6\times10^{5}$ | $3.41\times10^{-4}$ | 57 |
| XIII | Cyclohexyl | $6.1\times10^{4}$ | $3.07\times10^{-5}$ | >500 |
| XIV | Phenyl | $1.8\times10^{6}$ | $2.64\times10^{-3}$ | 2.8 |
| XV | Benzyl | $1.9\times10^{5}$ | $7.05\times10^{-4}$ | >500 |
| XVI | $ClCH_2CH_2CH_2$ | $5.9\times10^{5}$ | $7.53\times10^{-4}$ | 8.5 |
| XVII | $OC_2H_5$‡ | $2.7\times10^{7}$ | $6.14\times10^{-5}$ | 1.2 |

* Taken from Fukuto & Metcalf (30).

† The hydrolysis was extremely slow (1.5 per cent in 2 weeks), and an approximate value is given.

‡ XVII (para-oxon) is given so that the data of the phosphonates may be compared with those of a phosphate of similar structure.

out the remarkably good correlation between $K_e$ and fly toxicity. The relationship is shown in Figure 1. It is apparent from Table I and Figure 1 that, in spite of the complexity of insect intoxication, the toxicity of these compounds is directly related to the reactivity of the molecule, measured here by $K_e$ and $K_{hyd.}$. However, this relationship apparently does not hold true for alkyl *p*-nitrophenyl ethylphosphonates in which R in the alkoxy part of the molecule is varied, as shown in Table II. Although the premise that reactivity determines toxicity appears to hold true for the straight chain compounds, there is apparently little correlation between $K_e$, $K_{hyd.}$, and $LD_{50}$ for the branched compounds. For example, the *n*-decyl compound (XXII) and isopropyl compound (XXIII) have very similar values for $K_e$ and $K_{hyd.}$, yet their toxicities differ by a factor of 80. Hence, it appears that other factors, such as lipoid solubility, degradation rate, etc., must also be considered in insect intoxication.

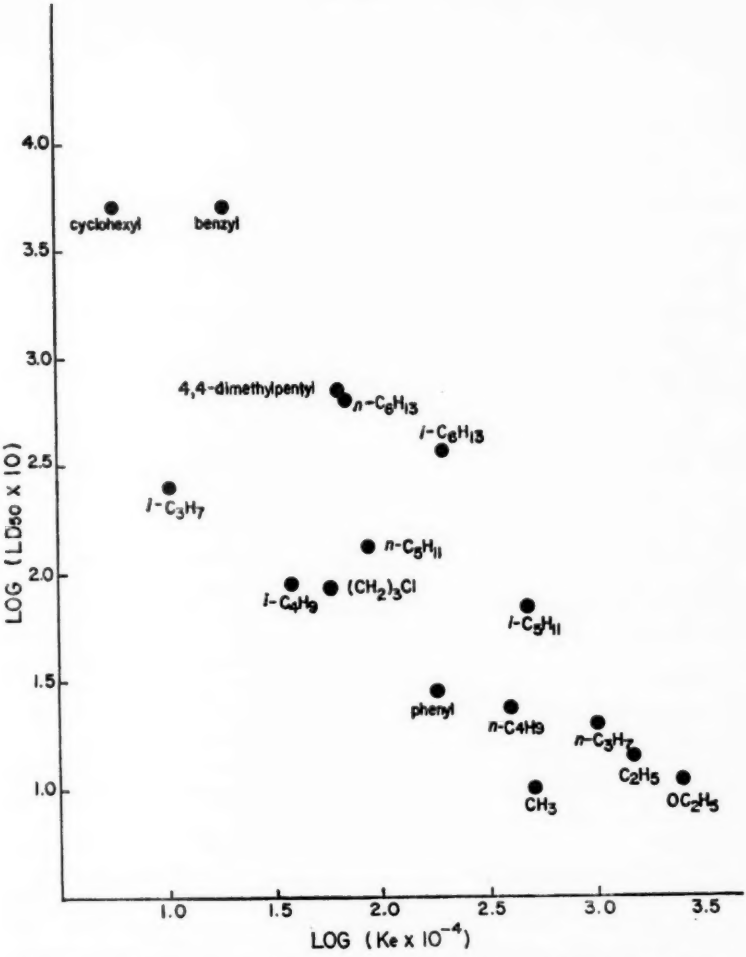



FIG. 1.—A plot of log cholinesterase inhibition constant $K_e$ versus log $LD_{50}$ to the common house fly. [Taken from Fukuto & Metcalf (30).]

TABLE II

RATE CONSTANTS AND TOXICITY DATA FOR ALKYL *p*-NITROPHENYL ETHYLPHOSPHONATES*

| | R | $K_s$ (1/moles/min.) | $K_{hyd.}$ (min.$^{-1}$) | $LD_{50}$ (μg./gm. fly) |
|---|---|---|---|---|
| XXVIII | $CH_3$ | $1.3\times10^{6}$ | $1.89\times10^{-3}$ | 1.2 |
| II | $C_2H_5$ | $1.5\times10^{7}$ | $1.28\times10^{-3}$ | 1.2 |
| XIX | *n*-$C_4H_9$ | $5.7\times10^{6}$ | $8.78\times10^{-4}$ | 1.45 |
| XX | *n*-$C_6H_{13}$ | $1.2\times10^{7}$ | $8.63\times10^{-4}$ | 5.35 |
| XXI | *n*-$C_8H_{17}$ | $1.3\times10^{7}$ | $8.42\times10^{-4}$ | 14.5 |
| XXII | *n*-$C_{10}H_{21}$ | $2.5\times10^{6}$ | $2.11\times10^{-4}$ | 120.0 |
| XXIII | *i*-$C_3H_7$ | $2.3\times10^{6}$ | $2.22\times10^{-4}$ | 1.5 |
| XXIV | *i*-$C_4H_9$ | $5.4\times10^{6}$ | $7.99\times10^{-4}$ | 1.05 |
| XXV | *s*-$C_4H_9$ | $2.5\times10^{6}$ | $2.83\times10^{-4}$ | 3.1 |
| XXVI | *i*-$C_5H_{11}$ | $4.2\times10^{6}$ | $8.29\times10^{-4}$ | 2.15 |
| XXVII | Neopentyl | $2.4\times10^{6}$ | $4.22\times10^{-4}$ | 2.0 |
| XXVIII | $(CH_3)_2CHCH(CH_3)$- | $1.6\times10^{6}$ | $1.60\times10^{-4}$ | 7.5 |
| XXIX | $(CH_3)_2CHCH_2CH(CH_3)$- | $1.6\times10^{6}$ | $1.49\times10^{-4}$ | 16.5 |
| XXX | $(CH_3)_3CCH_2CH_2$- | $5.4\times10^{7}$ | $9.44\times10^{-4}$ | 2.7 |

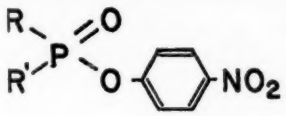
* Taken from Fukuto *et al.* (32, 33).

In addition to the compounds described above, some sulfur analogues of alkyl *p*-nitrophenyl alkylphosphonates were prepared and examined (33). *O*-Ethyl *O*-*p*-nitrophenyl ethylphosphonothionate (phosphonate analogue of parathion) was slightly more toxic to house flies ($LD_{50}$ 0.93 μg./gm. fly) than was the corresponding oxygen analogue. This compound was the most toxic compound to house flies found among the esters of phosphonic acid. Like parathion, it was stable to aqueous hydrolysis and showed no inhibition of cholinesterase, indicating that an activation process occurs in the animal, as has been shown to occur with parathion (34). In contrast, the *S*-ethyl and *S*-*p*-nitrophenyl esters of ethyl ethylphosphonic acid were rapidly degraded by water and were somewhat less toxic to house flies.

Kabachnik *et al.* (43) also reported a number of sulfur-containing phosphonate esters. Among the following compounds, methyl *p*-nitrophenyl methylphosphonothionate, ethyl *p*-nitrophenyl methylphosphonothionate, ethyl 4-methyl-7-coumaryl methylphosphonothionate (phosphonate analogue of Potasan® [*O,O*-diethyl *O*-(4-methylumbelliferone) phosphorothioate]), methyl *S*-(1,2-dicarbomethoxy)ethyl methylphosphonodithioate (similar to malathion), only the first compound was more active as a contact insecticide than its corresponding phosphorothionate ester.

Razumov and co-workers (75, 76) described a series of dialkylphosphinic

acid esters of *p*-nitrophenol of the structure shown below. Of these compounds, *p*-nitrophenyl dipropylphosphinate was found to be as strong an



$R = R' = CH_3$ to $C_4H_9$

inhibitor of cholinesterase as armin but 20 times less toxic to the white mouse. Fukuto *et al.* (33) have also synthesized a number of dialkylphosphinic acid esters of *p*-nitrophenol and determined their toxicities against house flies. Many were almost as toxic to the house fly as the corresponding phosphonate esters, as shown in Table III. The data show that phosphinates are generally more susceptible to aqueous hydrolysis than the phosphonate esters. In spite of the greater reactivity of phosphinate esters, they are generally poorer inhibitors of cholinesterase. The lower inhibition constants

TABLE III

RATE CONSTANTS AND TOXICITY DATA FOR *p*-NITROPHENYL DIALKYLPHOSPHINATES*

| | R | R′ | $K_i$ (1/moles/min.) | $K_{hyd.}$ (min.$^{-1}$) | $LD_{55}$ ($\mu$g./gm. fly) |
|---|---|---|---|---|---|
| XXXI | $C_2H_5$ | $C_2H_5$ | $2.8\times10^{6}$ | $1.87\times10^{-2}$ | 3.5 |
| XXXII | $C_2H_5$ | *n*-$C_3H_7$ | $2.1\times10^{5}$ | $1.51\times10^{-2}$ | 3.7 |
| XXXIII | $C_2H_5$ | *i*-$C_3H_7$ | $4.3\times10^{5}$ | $3.59\times10^{-3}$ | 6.5 |
| XXXIV | *n*-$C_3H_7$ | *n*-$C_3H_7$ | $7.1\times10^{5}$ | $1.51\times10^{-2}$ | 4.4 |
| XXXV | $C_2H_5$ | *t*-$C_4H_9$ | $1.7\times10^{4}$ | $4.67\times10^{-7}$ | 22.0 |
| XXXVI | *i*-$C_3H_7$ | *i*-$C_3H_7$ | $4.0\times10^{4}$ | $1.03\times10^{-3}$ | 65.0 |
| XXXVII | *n*-$C_4H_9$ | *n*-$C_4H_9$ | $1.4\times10^{5}$ | $1.33\times10^{-3}$ | 500 |
| XXXVIII | $C_6H_5$ | $C_2H_5$ | $7.9\times10^{6}$ | $6.40\times10^{-2}$ | 17.5 |
| XXXIX | $C_6H_5$ | $C_6H_5$ | $1.7\times10^{4}$ | $1.06\times10^{-1}$ | >500 |

* Taken from Fukuto *et al.* (33).

may be attributed to several reasons. Because of the high instability of these compounds, they may decompose before reacting with the enzyme, or the phosphorylated enzyme may be spontaneously hydrolyzed back to its original form. In spite of their lower anticholinesterase activity, many of the phosphinate esters were extremely toxic to house flies and approached the phosphonates in activity.

In addition to esters of *p*-nitrophenol, Metcalf & Fukuto (63) investigated the systemic activity of a variety of alkyl alkylphosphonate esters of

2-(ethylthio)ethanol and 2-(ethylthio)ethanethiol, groups present in the demeton isomers. These compounds were generally extremely effective as systemic insecticides against greenhouse thrips, *Heliothrips haemorrhoidalis* (Bouché), and two-spotted mites, *Tetranychus telarius* (Linnaeus). The most effective compounds were *O*-ethyl *S*-2-(ethylthio)ethyl ethylphosphonodithioate and the corresponding phosphonothiolate. Besides thrips and mites, these compounds controlled the larva of the cotton leaf perforator, *Bucculatrix thurberiella* Busck, by systemic action.

Of academic interest are the different activities shown by the optical isomers of *O*-ethyl *S*-2-(ethylthio)ethyl ethylphosphonothiolate. The optical antipodes of this compound may be prepared from the *dextro* and *levo* forms of *O*-ethyl ethylphosphonothioic acid (1, 2). It was found that the *levo* isomer inactivated cholinesterase from different sources 10 to 20 times faster than the *dextro* isomer. The *levo* isomer was 6, 10, and 9.7 times more toxic to the house fly, mosquito larva, and honey bee, respectively, than the *dextro* isomer (31).

Recently, the chemical structure of the compound *O*-methyl *O*-2,4-dichlorophenyl methylphosphonothionate was released by Farbenfabriken Bayer as compound 30911. Their preliminary evaluation indicates that this compound will be an extremely promising insecticide. Bayer 30911 is a relatively non-toxic phosphonate ester (oral $LD_{100}$ to rats, 500 mg./kg.) showing systemic, as well as contact, activity. It is particularly effective against a wide variety of cotton pests. In addition, it has demonstrated decided repellency to field mice and preliminary laboratory evaluation showed good activity against the root knot nematode, *Meloidogyne* spp. This compound is the first new phosphonate ester since EPN to reach the stage where it is being considered for commercial development.

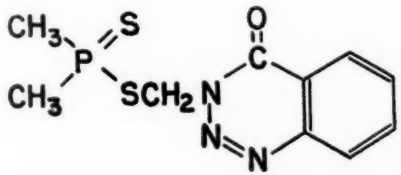
The possibilities of new compounds derived from dialkylphosphonic and dialkylphosphinic acids are enormous. From Tables I, II, and III, alone, one can estimate about 50 or more combinations of alkyl groups in the moieties (R'O)RP(O)- and R'RP(O)-, which when combined with an activating (or displaceable) group, such as *p*-nitrophenol, will give a highly insecticidal compound.

R and R' may be the same or different

The number of activating groups, X, appears to be limitless. Among the three dozen compounds cited to be in commercial or semicommercial use as of 1958 (61), there are some 23 different activating groups. The above phosphonate and phosphinate esters of these groups alone can give rise to 1250

potential compounds. By making the thionate analogues, this number is doubled.

The patent literature indicates that Schrader has recognized the potentialities of phosphonate and phosphinate esters as insecticides. Among the esters of phosphonic acid described as valuable insecticides under Bayer patents are *O*-ethyl *O*-(4-methylthiophenyl) methylphosphonate and methylphosphonothionate (24), *O*-ethyl *S*-2-(ethylthio)methyl methylphosphonothiolate (23), and others. More recently, a patent was filed by Schrader *et al.* (79) in which a large series of phosphinic acid esters and phosphinothioic acid esters of high insecticidal activity were described. Schrader points out that there is a definite need for new kinds of organophosphorus compounds, particularly in view of the development of resistance of various pests against esters of phosphoric acid. This patent reports the synthesis and some of the insecticidal properties of about 150 new phosphinate esters. It is significant that many of the phosphinothioic acid ester analogues of well-known commercial insecticides were considerably lower in mammalian toxicity than the corresponding phosphoric acid esters. For example, the acute oral $LD_{50}$ of Guthion® [*O,O*-dimethyl *S*-4-oxo-1,2,3-benzotriazin-3-(4*H*-ylmethyl) phosphorodithioate] to rats is 15 to 25 mg./kg. The dimethyl phosphinate analogue of Guthion, shown below, fed to rats at the dosage of 1000 mg./kg is reported to produce no symptoms of poisoning.

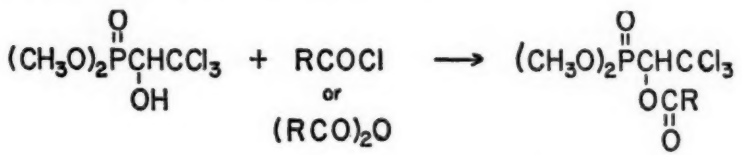


Another example is the relative toxicity of phorate [*O,O*-diethyl *S*-(ethylthio)methyl phosphorodithioate], $LD_{50}$ 3.7 mg./kg. (84), compared to the corresponding phosphinate, *S*-(ethylthio)methyl diethylphosphinodithioate, $LD_{50}$ 25 mg./kg. Among the other compounds described in this patent are the phosphinate analogues of the commercial insecticides Di-Syston® [*O,O*-diethyl *S*-2-(ethylthio)ethyl phosphorodithioate], demeton isomers, Trithion® [*S*-(*p*-chlorophenylthio)methyl *O,O*-diethyl phosphorodithioate], Tetram® [*O,O*-diethyl *S*-(β-diethylamino)ethyl phosphorothiolate], dimethoate, Phosdrin® (1-methoxycarbonyl-1-propen-2-yl dimethyl phosphate), parathion, Baytex® (*O,O*-dimethyl *O*-4-methylthio-3-methylphenyl phosphorothionate), and many others.

The chemistry and mechanism of action of Dipterex has attracted a great deal of attention. It is well-known that Dipterex rearranges to DDVP, under alkaline conditions (10, 54, 59). Arthur & Casida (6) have shown that the urine of female dogs treated with Dipterex contained 63 per cent of the

original compound in the form of trichloroethanol, indicating that cleavage of the carbon-phosphorus bond occurs primarily in mammals. Metcalf & Fukuto (62) and Miyamoto (66) independently investigated the rate of conversion of Dipterex to DDVP under different pH conditions. By chloride ion and radiotracer analysis, Metcalf *et al.* (62) found that even at pH 6.0 Dipterex rearranged slowly to DDVP and at pH 7.0 and 8.0 the rearrangement was quite rapid. The half life of Dipterex at pH 7.0 was 386 minutes. These findings were corroborated by Miyamoto, who followed the reaction by a manometric method. Miyamoto showed that Dipterex did not inhibit chymotrypsin at pH 5.0, while, under the same condition, DDVP showed some inhibition. Metcalf *et al.* showed the same to hold true for fly brain cholinesterase. From this and other substantiating evidence, both workers concluded that the toxic action in house flies was exerted through the *in vivo* formation of DDVP.

A clever modification of the Dipterex molecule was made by Arthur & Casida (7), who examined the biological properties of acylated Dipterex prepared according to the following reaction.




They prepared compounds in which the R group above was varied from methyl to *n*-heptyl. The acylated compounds were remarkably less toxic to rats than Dipterex and still showed considerable toxicity to the house fly. The best compound in the series was dimethyl 2,2,2-trichloro-1-*n*-butyryloxyethyl phosphonate ($R = n\text{-}C_3H_7$). This compound, called butonate, showed the same toxicity to house flies as did Dipterex but was more than 7.5 times less toxic to the rat. The same carboxylic esters of the diethyl analogue of Dipterex, diethyl 2,2,2-trichloro-1-hydroxyethylphosphonate, were considerably more toxic to house flies than the parent compound, but the most active ones were also more toxic to the rat. The relative mammalian toxicities shown by the dimethyl and diethyl analogues indicate that in acylated Dipterex the *in vivo* formation of DDVP is slowed down to such an extent that demethylation becomes the primary degradation mechanism. It has been shown that catalytic demethylation of trimethyl phosphoric acid esters occurs much faster than de-ethylation of the corresponding triethyl ester (21). In fact, de-ethylation proceeds with difficulty. Thus, acylation can help degradation via dealkylation in Dipterex but not in the case of the diethyl analogue. Nikoronov (71), in addition to some of the compounds described above, reacted Dipterex with dialkyl phosphorochloridate and obtained the corresponding phosphorylated products. He reported that these compounds showed some systemic insecticidal activity.

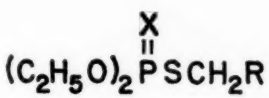
### Phosphoric Acid Esters

Space limitations prevent a complete discussion of the new compounds in this group. As mentioned earlier in the introductory remarks, many of the commercially important esters of phosphoric acid have been the subject of numerous review articles.

The single organophosphorus insecticide produced in largest quantity today is probably malathion. The remarkable success of malathion may be attributed to its low mammalian and good insect toxicity. This differential toxicity between insects and mammals has been attributed to more extensive degradation in the mammal than in the insect (56). Mouse liver has been shown to rapidly degrade malathion and its oxygen analogue, mala-oxon, and this degradation has been shown to occur at the carboxylic ester bond (72). In addition, low and transient levels of mala-oxon were found in the mouse while high and persistent levels were found in two malathion-susceptible insects (49).

Recently, other compounds have appeared that structurally resemble malathion and also show differential toxicity to insects and mammals. The most prominent compound of this group is dimethoate [*O,O*-dimethyl *S*-(N-methylcarbamoylmethyl) phosphorodithioate]. This compound was reported as early as 1950 by Cassaday *et al.* (17) and was initially developed some years later by "Montecatini" in Italy (69). Dimethoate is a white crystalline solid, m.p. 51°–52°C., and soluble in water to 7 per cent. The acute oral $LD_{50}$ to rats is 245 mg./kg. (20). The uptake and metabolism of dimethoate in plants and animals is rapid and, in gross aspects, appears to be the same as malathion (19, 20, 44). The metabolic routes proposed for dimethoate are very similar to those proposed for malathion (56). Curiously enough, dimethoate and its oxygen analogue are relatively poor inhibitors of red blood cell cholinesterase, $I_{50}$ $2.5\times10^{-1}$ *M* and $1.2\times10^{-4}$ *M* (44). This compound is one of the most effective known against house flies with a $LD_{50}$ of 0.43 μg./gm. fly (50). However, the $I_{50}$ value for house-fly head cholinesterase by dimethoate oxygen analogue has been reported to be $9.6\times10^{-7}$ *M* (44), and this is in line with the toxicity of dimethoate to flies. The toxicity differential between the white mouse and insects has been attributed to the more rapid degradation of dimethoate or its oxygen analogue by the mouse than by insects (50).

Reasoning that in compounds of the malathion type hydrolysis of the carboxylic ester moiety to the acid leads to a lowering in toxicity and that this reaction occurs faster in mammals than in insects, O'Brien *et al.* (73) prepared and examined a series of compounds of the type below, where R is a carboxylic ester moiety.



The outstanding compound in this series was acethion ($X=S$, $R=CO_2C_2H_5$), which showed even greater differential toxicity to the white mouse and house fly than did malathion. The $LD_{50}$ to the white mouse and house fly was 1280 mg./kg. and 9.4 μg./gm. fly, respectively, thus successfully supporting his rationale.

## Carbamic Acid Esters

The widespread success of the carbamate insecticide Sevin® (1-naphthyl-*N*-methylcarbamate), in controlling certain types of pests has created a great deal of interest in compounds of this type. Although the insecticidal potential of carbamates had been recognized for many years (36, 37, 40), it was not until the work of Kolbezen *et al.* (48), who described the insecticidal properties of a large series of simple substituted phenyl *N*-methylcarbamates, and the closely following discovery of Sevin that intensive research with these compounds has been carried out.

The early work of the Geigy Chemical Corporation, which apparently began in 1947, described several rather exotic *N,N*-dimethylcarbamates with outstanding insecticidal properties. These compounds, dimetan (*N,N*-dimethylcarbamate of 5,5-dimethyldihydroresorcinol), Pyrolan® (1-phenyl-3-methylpyrazolyl-5 dimethylcarbamate), Isolan® (1-isopropyl-3-methylpyrazolyl-5 dimethylcarbamate), and Pyramat® (2-*n*-propyl-4-methylpyrimidyl-6 dimethylcarbamate) are highly toxic to house flies and other insects, but generally ineffective against mites.

Kolbezen *et al.* (48) investigated a large series of substituted phenyl *N*-methylcarbamates in an attempt to correlate chemical structure with fly brain cholinesterase inhibition and insecticidal activity. For the monosubstituted phenyl *N*-methylcarbamates, it was found that *in vitro* cholinesterase inhibition was directly related to the stability of the compound to aqueous hydrolysis. The order of effectiveness of *m*- and *p*-monosubstituents on cholinesterase inhibition was $(CH_3)_3N^+ > tert\text{-}C_4H_9 > (CH_3)_2N > iso\text{-}C_3H_7 > C_2H_5 > CH_3 > Cl > NO_2$. The order of activity, with the exception of the trimethylammonium group, indicates that the anticholinesterase activity is a function of the electron-donating capacities of the substituents toward the *N*-methylcarbamoyl moiety. The increased electron density around the carbonyl function tends to stabilize the molecule to hydrolytic attack. Conversely to the organophosphorus compounds, one of the structural requirements for high anticholinesterase activity by a carbamate is that it be stable to hydrolysis and, thus, behave as a competitive inhibitor and not as a substrate of cholinesterase. In addition to hydrolytic stability, another important structural requirement for high anticholinesterase activity is that the molecule "fit well" on the enzyme surface at the active site and be able to block the normal substrate reaction. This requirement is partially fulfilled by the *N*-methylcarbamoyl moiety, which presumably is attracted to the esteratic site by the same forces that bind the acetoxy moiety of acetyl cho-

line. This is evident from Kolbezen's data, which show that, in general, those compounds that possess stable, unhindered *N*-methylcarbamoyl moieties were, to varying degrees, inhibitors of cholinesterase. In addition, anticholinesterase activity can be enhanced by substituents in the *o*- and *m*-positions, which spatially resemble the trimethylammonium group of acetyl choline, such as the *m*-dimethylamino, *m*-isopropyl, *o*- and *m-t*-butyl groups. Thus, to some degree, it is possible to predict the anticholinesterase activity of monosubstituted phenyl *N*-methylcarbamates from the spatial configuration of the molecule and electronic properties of the substituents.

The relationship between anticholinesterase activity and insect toxicity is somewhat more complex. Although Kolbezen *et al.* (48) were able to demonstrate good correlation between cholinesterase inhibition and toxicity to greenhouse thrips, *H. haemorrhoidalis*, no correlation was found between inhibition and house fly toxicity. Kolbezen's data and subsequent data by Metcalf *et al.* (65) indicate that toxicity to house flies is dependent on the rate of *in vivo* detoxification of the carbamate. This is discussed in the following paragraphs.

One interesting aspect of the carbamate insecticides is the enhancement of toxicity shown when they are used together with pyrethrin synergists. Moorefield (67) demonstrated that the toxicity of Sevin, Pyrolan, and Isolan to house flies, German cockroach [*Blatella germanica* (Linnaeus)], and bean aphid (*Aphis fabae* Scopoli) was enhanced when each of these carbamates was used together with any of the methylenedioxyphenyl synergists, piperonyl butoxide (3,4-methylenedioxy-6-propylbenzyl *n*-butyl diethylene glycol ether), Sesoxane® [2-(3,4-methylenedioxyphenoxy)-3,6,9-trioxaundecane], sulfoxide (*n*-octylsulfoxide of isosafrole), *n*-propyl isome (di-*n*-propyl 6,7-methylenedioxy-3-methyl-1,2,3,4-tetrahydronaphthalene-1,2-dicarboxylate), and sesamine oil derivatives. The highest degree of carbamate synergism was obtained with piperonyl butoxide, Sesoxane, and sulfoxide; e.g., 0.2 μgm. Sevin and 2.0 μgm. piperonyl butoxide per fly (non-resistant NAIDM strain), used separately, were completely ineffective but, when applied in combination, resulted in 100 per cent mortality. Synergism of Sevin by Sesoxane has also been demonstrated against DDT-resistant flies (22).

Moorefield (68) made some interesting observations on the development of resistance to carbamate insecticides by house flies. Laboratory selection of house flies with Sevin and *m-t*-butylphenyl *N*-methylcarbamate, two relatively ineffective compounds, rapidly produced flies (10 generations) completely resistant to these carbamates. In contrast, *m*-isopropylphenyl *N*-methylcarbamate, which is some three to four times more toxic to flies, developed only eightfold resistance after 67 generations. However, resistance did not develop when flies were selected with a combination of Sevin and piperonyl butoxide. In addition, the per cent mortality of susceptible flies treated with either Sevin or *m-t*-butylphenyl *N*-methylcarbamate never exceeded 70 per cent, in spite of extremely high dosages. The *in vitro* fly head

anticholinesterase activity of the *m*-isopropylphenyl, *m-t*-butylphenyl compounds, and Sevin were virtually the same, ranging from $I_{50}$ values of 1.5 to $3.7\times10^{-7}$ *M*.

The findings expressed in the two preceding paragraphs strongly indicate that the intoxication of an insect by a carbamate is dependent on its rate of detoxification in the insect. It appears that resistance development is caused by an increase in the detoxification rate. Although Moorefield (68) did not find any evidence of *in vitro* enzymatic breakdown of Sevin by house fly head or whole fly breis, later work by Georghiou & Metcalf (see 38) has shown that *m*-isopropylphenyl *N*-methylcarbamate is degraded *in vivo* in house flies and that the degradation rate is suppressed by a synergist. Also, it was found that the degradation rate is faster in carbamate-resistant flies than in the susceptible strain. Subsequent work by Metcalf *et al.* (65) has further demonstrated the importance of detoxification in insect intoxication by carbamates. These workers examined a large series of mono-, di-, and trisubstituted alkoxyphenyl *N*-methylcarbamates for insect toxicity and fly brain cholinesterase inhibition. Some of their data is presented in Table IV.

TABLE IV

FLY BRAIN ANTICHOLINESTERASE ACTIVITY AND HOUSE FLY TOXICITY DATA OF SOME SUBSTITUTED ALKOXYPHENYL *N*-METHYLCARBAMATES*

| Substituent | $I_{50}$ (*M*) | $LD_{50}$ (μg./gm. fly) |
|---|---|---|
| 2,3-di-$CH_3O$ | $1.4\times10^{-5}$ | >500 |
| 2,4-di-$CH_3O$ | $2.8\times10^{-5}$ | 155 |
| 2,5-di-$CH_3O$ | $1.3\times10^{-5}$ | 13 |
| 2,6-di-$CH_3O$ | $2.1\times10^{-3}$ | >500 |
| 3,4-di-$CH_3O$ | $1.9\times10^{-5}$ | 400 |
| 3,5-di-$CH_3O$ | $8.0\times10^{-6}$ | 11 |
| 3,4,5-tri-$CH_3O$ | $6.5\times10^{-6}$ | 31 |
| 3,4-methylenedioxy | $1.3\times10^{-4}$ | 17.5 |

* Taken from Metcalf *et al.* (65).

The data show that, with the exception of the 2,6-dimethoxy compound, in which the *N*-methylcarbamoyl moiety is obviously well shielded by the methoxy groups and the 3,4-methylenedioxy compound, all compounds show about the same $I_{50}$ values. The wide variation in toxicity of the various positional isomers is most striking, particularly the difference in activity shown by the 3,4-dimethoxy and 3,5-dimethoxy compounds. However, when piperonyl butoxide was used in combination with each of these two compounds (ratio of 1 part carbamate to 5 parts piperonyl butoxide) the $LD_{50}$ value of the 3,4-compound and 3,5-compound was 12.00 and 4.45 μg./gm. of fly. The degree of enhancement of toxicity by piperonyl butoxide for the 3,4-compound is 33 (400/12.00), compared to 2.5 (11/4.45) for the 3,5-com-

pound, and this large difference in synergistic activation can only be logically explained by the suppression of the *in vivo* detoxification rate of the 3,4-compound so that it is permitted to reach a vital center. The ratio of $LD_{50}$ values of the 3,4- and 3,5-compounds with piperonyl butoxide (12.0/4.45 = 2.7), then, is in good agreement with the ratio of their anticholinesterase activity ($19\times10^{-5}/8\times10^{-5}=2.4$). Thus, the different relative toxicities of the substituted phenyl *N*-methylcarbamates on house flies and greenhouse thrips (48) is probably attributable to differences in the ability of these insects to detoxify these compounds. The linear relationship found by Kolbezen *et al.* (48) for cholinesterase inhibition and toxicity with the greenhouse thrips indicates that a rapid detoxification mechanism is absent in this insect.

Very little work has been done on the mode of detoxification of carbamates in biological systems. It has been shown that aromatic and aliphatic esterases and cholinesterases from various sources do not catalyze the hydrolysis of Sevin to 1-naphthol (16). By electrophoretic separation, it was possible to show that plasma albumin was capable of liberating naphthol from Sevin. It is obvious that much work is needed to elucidate the nature of these detoxifying enzymes in order to develop active compounds that are not easily detoxified and for the development of superior synergists.

At the present time, Sevin is the only carbamate that has attained commercial importance in the United States. The pure material is a white crystalline solid, m.p. 145°C., vapor pressure of less than 0.005 mm.Hg. at 26°C., and a density of 1.232 at 20°C. (41). Results of acute toxicity tests indicate an oral $LD_{50}$ for rats in the range of 500 to 700 mg./kg. Sevin has been found to be highly effective in controlling a wide variety of insects, including the apple aphid and other apple pests (35), codling moth (55), corn earworm (5), various cotton insects (11), variegated grape leafhopper (8), Mexican bean beetle (12), and other insects too numerous to mention here.

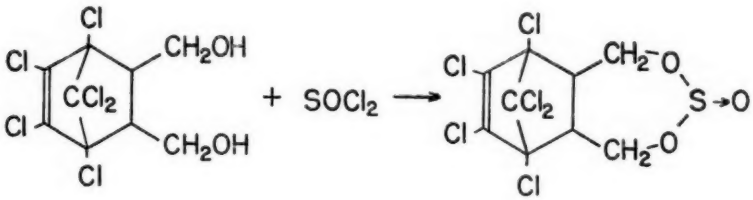
There are numerous other carbamate insecticides now undergoing experimental evaluation. These include DOWCO-139, Rohm & Haas C-140 (dimethyl carbamate of 2-dimethylaminomethylphenol, hydrochloride), Hercules *m*-isopropylphenyl N-methylcarbamate, and Bayer 39007. Some of these appear to be highly promising and are expected to develop into commercially important compounds.

## Organochlorine Compounds

The spectacular success of such compounds as chlordane, heptachlor, aldrin, dieldrin, and others in insect eradication since the insecticidal activity of technical chlordane was first reported in 1945 (46, 47) has stimulated the search for insecticides of the chlorinated bicyclodiene type. Some of the more recent compounds possessing the fundamental bicycloheptadiene structure are reported here.

*Thiodan*®.—Thiodan (6,7,8,9,10,10-hexachloro-1,5,5*a*,6,9,9*a*-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide) was discovered by Farbwerke

Hoechst AG in Germany (28) and is presently being developed in the United States by the Niagara Chemical Division, Food Machinery and Chemical Corporation. This compound has been registered for the control of a wide variety of insects and mites on many kinds of crops (82). The technical material consists of two isomers with similar insecticidal properties (26, 53). Thiodan is prepared by refluxing a mixture of Thiodan alcohol and thionyl chloride.

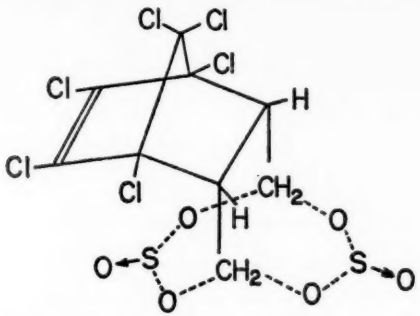



Thiodan alcohol Thiodan

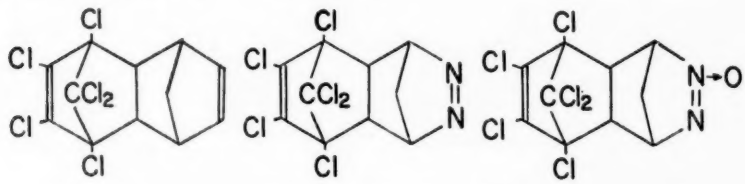
Purified Thiodan, m.p. 93°–95°C., has been separated into two stereoisomers from an aluminum oxide column; isomer A, m.p. 108°–109°C., and isomer B, m.p. 206°–208°C. in the ratio of 4 to 1. The actual configurations of isomers A and B have not been worked out. Lindquist & Dahm (53) have done some interesting work in this regard. When either isomer, A or B, is subjected to acidic hydrolysis, a Thiodan alcohol is generated. Melting point data, paper chromatographic analysis, and infrared spectra indicated that only one alcohol was produced from the two isomers. However, when the alcohol from A or B was refluxed with thionyl chloride, each gave a mixture of isomers A and B. The most reasonable explanation for these findings is the following. Thiodan alcohol is prepared from hexachlorocyclopentadiene and 1,4-dihydroxy-2-butene (27) and can theoretically exist in three different forms, *endo-cis*, *exo-cis*, and *trans*. It has been shown that, when cyclopentadiene reacts with maleic anhydride in a Diels-Alder reaction, the *endo* configuration is produced exclusively (3, 4). Also, it has been calculated that the attracting forces between two molecules are greater in the *endo* than in the *exo* orientation (87). When the dienophile has no activating unsaturation (as in allyl alcohol, allylamine, etc.), the orientation of the two molecules to give the *endo* configuration is attributed to the unshared electrons on oxygen or nitrogen. Thus, by analogy with cyclopentadiene and a dienophile, Thiodan alcohol is probably *endo-cis*, since the *trans* isomer can give *d-l* isomers only. The isomeric compounds, A and B, therefore, must arise from the *endo* or *exo* configuration of the seven-membered sulfite ring, as shown below.

The Fisher-Hirschfelder model of the seven-membered sulfite ring shows that the two configurations are rigid when two adjacent bonds are fixed.

***Chlorinated bridged phthalazines.***—The high insecticidal activity of a number of chlorinated bridged phthalazines is of interest because of their



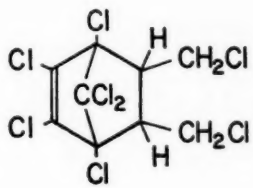
structural relationship with aldrin and dieldrin (51, 52). The phthalazine analogue of aldrin (5,6,7,8,9,9-hexachloro-1,4,4*a*,5,8,8*a*-hexahydro-1,4,5,8-dimethanophthalazine) and its *N*-oxide are reported to be 40 and 50 times, respectively, more toxic than DDT toward the common house fly. Their structures are shown below with aldrin.



Aldrin Phthalazine analogue *N*-Oxide of phthalazine analogue

The *N*-oxide was prepared by peroxyacetic or peroxybenzoic acid oxidation of the dimethanophthalazine. The stereochemistry of these compounds has not been studied, but, since they were prepared by the reaction of hexachlorocyclopentadiene with a bicycloheptene double bond, as is aldrin, they are assumed to have the same configuration. Aldrin is in the *exo, endo* configuration.

*Other organochlorine compounds.*—Another Farbwerke Hoechst compound related to Thiodan and of special interest because of its low mammalian toxicity is Alodan®, 5,6-*bis*-(chloromethyl)-1,2,3,4,7.7-hexachlorobicyclo-2,2,1-heptene-2.



The oral $LD_{50}$ of this compound is reported to be 15 gm./kg. (18, 42). Because of its low toxicity, this compound has been shown to be extremely useful for the eradication of ectoparasites. Hohorst & Bauer (42) have shown that in tests with 37 different ectoparasites this compound was effective against all except six of them.

Another chlorinated cyclodiene compound reported to have excellent insecticidal properties is 4,5,6,7,10,10-hexachloro-4,7-endomethylene-4,7,8,9-tetrahydrophthalan (25), prepared from the Diels-Alder reaction of hexachlorocyclopentadiene and 2,5-dihydrofuran.

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# MODE OF ACTION OF INSECTICIDES[1,2,3]

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This topic has been the subject of several reviews and symposia in the past two years (10, 68, 69, 76). Although the term "mode of action" and its scope have not been clearly defined or agreed upon, several workers have set forth their views on the subject. In their recent review, Winteringham & Lewis (78) comment on the difficulties inherent in a search for a single mode or mechanism. In dealing with the mode of action of insecticides we are, by the definition of the word "insecticide," seeking the cause or causes of the death of the insect. Spiller (63) states:

> If "to kill" is "to cause the death of" then insecticides kill insects. But the word that should be operative here is "cause" rather than "death" for there may be a rather long and at first not very obvious chain of events, beginning with the observed biochemical or biophysical effects of an insecticide on some particular system and ending with the death of the insect.

Beard (5) comments on the diverse connotations of the term "mode of action," particularly in contrast to Metcalf's (48) statement of the more specific concept relating the mode of action of poisons to specific interference with biochemical systems, largely enzyme in nature.

Jochum (37) defines the problem as follows:

> In order to provide an answer to the question as to the cause of death and also the site of action by the poison, it is important to establish in what order, at what rate and in what manner each pathological change occurs and spreads.

All of these definitions appear to imply some considerable knowledge of the normal processes of the experimental animal. A fundamental prerequisite to an understanding of these phenomena, both normal and pathological, is a greatly enhanced effort in the areas of comparative physiology and biochemistry. Reviews by Winteringham (76) and Winteringham & Barnes (77) indicate something of the nature of the progress in these areas.

We propose to consider mode of action in a broad concept, seeking some relationship in the gross symptoms observed in intoxicated animals and the more specific physiological changes that have been reported. Specifically, we will consider three phases, i.e., symptomatology, neurosecretion, and intermediary metabolism, as they are affected by insecticidal exposures and other environmental stresses that may produce analogous effects.

[1] The survey of the literature pertaining to this review was concluded in April, 1960.

[2] The common names and abbreviations for insecticides in this chapter follow the usage of the Committee on Insecticide Terminology of the Entomological Society of America, as presented in the *J. Econ. Entomol.*, **52**, 361–62, 1032 (1959).

[3] Contribution No. 772, Department of Entomology, Kansas Agricultural Experiment Station, Manhattan, Kansas.

The importance of neurosecretions in regulating physiological activity and behavior of the insect suggest that we examine this area to establish their potential interactions with insecticide intoxication and other environmental stresses. A summary of the action of neurosecretions on various physiological activities is presented in Table I. This summary indicates the

TABLE I

THE EFFECTS OF NEUROSECRETORY MATERIAL ON PHYSIOLOGICAL ACTIVITY IN INSECTS

| Physiological area | Neurosecretory source | Species | Effects observed | Reference |
|---|---|---|---|---|
| Behavior | Corpus cardiacum | *Periplaneta americana* (Linnaeus) | In-co-ordination, stereotyped locomotor behavior | 57 |
| | Subesophageal ganglion | *P. americana* | Activity rhythm | 32 |
| Reproductive organs | Brain | *Carausius morosus* Brunner | Increased oviduct movement | 24 |
| | Brain* | *Iphita limbata* Stål | Oviposition stimulated | 51 |
| | Corpus allatum | *C. morosus* | Depressed oviduct movement | 24 |
| Alimentary tract | Brain | *C. morosus* | Depressed gut movement, Malpighian tubules stimulated | 24 |
| | Corpus cardiacum | *P. americana* | Hind-gut stimulated, foregut inhibited | 15 |
| | Corpus allatum | *C. morosus* | Stimulated gut movement | 24 |
| Water regulation | Brain | *Anisotarsus cupripennis* Germar | Promoted $H_2O$ retention | 52 |
| | Corpus cardiacum | *A. cupripennis* | Promoted $H_2O$ retention | 52 |
| | Corpus cardiacum | *Apis mellifera* Linnaeus | Decreased $H_2O$ excretion | 1 |
| | Corpus allatum | *A. mellifera* | Increased $H_2O$ excretion | 1 |
| Blood | Corpus cardiacum | *A. mellifera* | Increased blood viscosity | 1 |
| | Corpus allatum | *A. mellifera* | Decreased blood viscosity | 1 |
| Heart | Corpus cardiacum | *P. americana* | Increased beat frequency | 20 |
| | Corpus cardiacum | *P. americana* | Increased beat frequency and amplitude | 15 |
| Spontaneous nerve activity | Brain | *P. americana* | No effect | 57 |
| | Corpus cardiacum | *P. americana* | Depressed† | 57 |
| | Corpus cardiacum | *P. americana* | Stimulated at low concentrations, depressed at high concentrations | 50 |
| | Corpus cardiacum | *P. americana* | Stimulated | 20 |
| | Corpus allatum | *P. americana* | No effect | 20 |
| | Corpus allatum | *P. americana* | No effect | 57 |
| | Abdominal ganglia‡ | *P. americana* | Stimulated at low concentrations, depressed at high concentrations | 62 |
| | Abdominal ganglia | *P. americana* | No effect | 57 |

* Medial neurosecretory cells.

† Rare cases where low concentrations increased activity.

‡ Central nervous system excited electrically.

broad scope of neurosecretory control and action. Although we concede the hazards implicit in suggesting cause-and-effect relationships, we do propose to emphasize the analogous phenomena reported in the various areas.

## Symptomatology

The post-treatment period may be conveniently divided into six stages: I, normal activity; II, hyperactivity; III, lack of co-ordination; IV, prostration and convulsions; V, general paralysis; and VI, apparent death. The latent period between the exposure and discernible symptoms of intoxication, as well as the duration of the various stages, appear to be, in part, characteristic of the species of animal and toxicant and are further controlled by the manner, amount, and conditions of exposure. Stages II, III, IV, and V have been observed in part or *in toto* for many insect and insecticide combinations.

The following examples illustrate the typical or atypical gross symptoms induced by several classes of organic insecticides during these stages. Hyperactivity and rapid paralysis of house flies exposed to pyrethrins is well-known. Busvine (14) described a characteristic fanning action of the wings of house flies following exposure to the cyclodiene insecticides. Chadbourne & Rainwater (16) recorded an inactive period for *Heliothis zea* (Boddie) after exposure to DDT or dieldrin, followed by tremors and convulsions which become severe after several hours. Beard (6) described a syndrome of symptoms for *Galleria mellonella* (Linnaeus) larvae poisoned with DDT in which prostration is typically absent and muscular activity continuous until death. Under certain conditions, however, the larvae become prostrate when regurgitated crop contents contaminate the integument. Jochum (37) has treated the symptoms of *Leptinotarsa decemlineata* (Say) intoxicated with parathion in considerable detail. During the latent period he sometimes observed a period of quiescence followed by the usual excitation progressing to the prostrate status that usually preceded death. Jochum makes the following generalization: "The more agile an insect is under natural conditions the more pronounced are the abnormal movements when poisoned and in a state of excitation." The generalized symptomatology appears to hold true, with the exception of *Ryania* alkaloids (34), which produces a paralysis without the preliminary excitation stage. Hassett (34) also reports that *Periplaneta americana* (Linnaeus) poisoned with ryanodine do not respond to mechanical or electrical activity.

Stresses other than insecticide intoxication appear capable of eliciting gross symptoms analogous to those discussed previously. Beament (4) has observed an ultimate prostration of *P. americana* resulting from mechanical restraint. The imposition of this restraint induces a hyperactive reaction in the form of struggling prior to prostration. Beament also applied mechanical and electrical stimulation to the test insect free of restraint and observed an ultimate prostration not unlike that produced by DDT intoxication. Tauber *et al.* (70) observed that *Melanoplus bivittatus* (Say) became very nervous and reacted to mild stimuli when subsisting on a dry food rather than the preferred succulent diet.

An inspection of Table I shows that at least two neurosecretory sources have been found to be associated with activity and behavior. The extent of the potential involvement of these secretions will become more apparent as our discussion progresses.

*Oviposition and copulation.*—A more specific phenomenon associated with intoxication is apparent in Jochum's (37) observation of the behavior of *L. decemlineata* poisoned with parathion. "The male insects increase their attempts at copulation, and the female beetles deposit several eggs but not in the usual manner." Atypical oviposition is frequently observed during the course of response to insecticides. Gravid female *Musca domestica* Linnaeus have been observed by the authors to extrude numerous eggs during stages III and IV after treatment with a variety of organophosphorus insecticides or during the use of $CO_2$ anaesthesia in experimental routines. Whitney (unpublished data, this laboratory) has noted an apparent stimulation of oviposition by *Tribolium confusum* Duval exposed to a variety of fumigant materials. Parker & Campbell (58) have observed that intoxication with pyrethrum caused a premature drop of the ootheca of female *Blattella germanica* (Linnaeus).

Other environmental stresses may also be the cause of excessive or atypical oviposition. Whitney (personal communication) has observed such phenomena in *T. confusum* subjected to radiofrequency irradiation at sublethal levels. The senior author has noted that female Madeira roaches, *Leucophaea maderae* (Fabricius), receiving lethal or sublethal exposures to the stress of radiofrequency radiations extruded the ootheca immediately upon removal from the radiation field. In situations where the exposure periods were of sufficient duration, the process of extruding the egg mass was initiated during the irradiation.

It has been demonstrated that brain extracts, when injected into insects, are capable of inducing oviduct movement and oviposition while corpora allata extracts had the opposite effect (Table I).

*Regurgitation, defecation, and water regulation.*—Other phenomena frequently associated with intoxication are excessive regurgitation and defecation. In this respect, Beard (6) has observed the typical DDT prostration in *Galleria* larvae only if contamination of the larvae with regurgita has occurred during the hyperactive stage. This prostration was not an irreversible prelude to death. If the regurgitated gut contents were prevented from being smeared over the larvae, prostration was not observed, even though death was the ultimate result of intoxication. Muscular contractions of the foregut after injection with DDT, possibly reinforced by body wall movements, were the cause of regurgitation. Clarke & Grenville (18) have demonstrated that movements of the foregut of *Schistocerca gregaria* (Forskål) are under the autonomous control of the ventricular (ingluvial) ganglion.

Chadbourne & Rainwater (16) reported that, during the tremor stage, the dieldrin-intoxicated larvae of *H. zea* were wet with a liquid that came from the mouth and anus and, apparently, through the integument of the body wall. In the later stages of convulsions, these authors observed the hind-

gut tissue of these larvae protruding through the anal opening by several millimeters. In the case of parathion-intoxicated *L. decemlineata*, Jochum (37) has reported a general defecation and the discharge of a considerable quantity of orange-red liquid from the mouth opening. Both *L. maderae* and *P. americana* intoxicated with a variety of organophosphorus insecticides have been observed by the authors to void a liquid excrement rather than the usually observed formed, dry pellets. Buck *et al.* (13), by use of ligation experiments, have reported that a small, though statistically significant, amount of weight was lost by regurgitation of *Phormia regina* (Meigen) larvae intoxicated with DDT. Ludwig (43) has observed that DDT-poisoned larvae of *Popillia japonica* Newman eliminate a considerable quantity of fecal material during the period of hyperactivity.

The water regulation of the experimental animal is apparently correlated, in part, with the phenomena of regurgitation and defecation. Buck & Keister (12) observed that DDT poisoning in adult *P. regina* increased both the reserve and metabolic water loss and that the former was several times the latter. In these experiments, the authors concluded that, in view of a lack of defecation, the only pathway for the water loss was by increased transpiration. In a later publication, these authors (13) attributed the major weight losses in the larvae to water losses. Jochum (37) attributed the majority of the weight losses observed in parathion-poisoned adult *L. decemlineata* and larval *Bombyx mori* (Linnaeus) to water losses, primarily by regurgitation. Ludwig (43) reported a loss of weight in DDT-poisoned adult and larva *P. japonica*. Under average conditions of relative humidity, the percentage decrease in total water is not different from the control insects. Ludwig did observe a greater water loss in those poisoned insects maintained over $CaCl_2$. This author states that the DDT appears to alter the ability of the larvae to resist desiccation. Dahm & Kearns (23) reported a significant weight loss in DDT-poisoned *M. domestica* but could not attribute this to water losses.

The loss of water by an intoxicated insect may be viewed from another aspect. Although there may not be a total loss of water from the insect as an entity, there have been observations indicating changes in the viscosity of the blood and gut contents, suggestive of an altered distribution of available water. Jochum (37) refers to a pronounced atrophy of the body cavity fluid observed in insects poisoned with parathion. Roan *et al.* (60) observed an apparent lack of blood in *P. americana* prostrate with poisoning from intoxication with organophosphorus compounds. Chamberlain & Hoskins (17) reported a similar phenomenon for parathion-intoxicated *P. americana*. Tobias *et al.* (71) commented on the appearance of dehydration when dissecting DDT-prostrated *P. americana*. Jochum (37) reported another phenomenon concerned with water regulation. He observed that the gut of *B. mori* filled perceptibly on intoxication with parathion. Jochum also presented data on the change in the specific gravity of the blood of parathion-poisoned *Dendrolimus pini* (Linnaeus); these data indicate an increase in the specific gravity of the blood from poisoned larvae and a corresponding decrease in the water content of the blood. Beament (4) has some very interesting observa-

tions concerning apparent blood volume or viscosity and other conditions involving water control in an insect subjected to environmental stresses. After 10 to 15 days of paralysis, the amount of hemolymph is markedly reduced. Beament does not find this observation surprising in view of the prolonged period of deprivation of food and water. A further observation of Beament's is of interest:

> . . . the impression of water depletion suggested by the hemolymph is not obvious in other body systems. In particular the hind gut seems to contain an abnormally large quantity of fluid compared with a desiccated but otherwise normal control. Even if one assumed that the feces had been retained because of failure of the muscular or nervous control of the rectum, it was obvious that, also, the water resorbing mechanism which concentrates the feces had not been functioning properly.

Reference to the summary of neurosecretory actions on control of muscular movement of the alimentary tract, water regulation, excretion, and blood viscosity will indicate further the possibilities of their involvement in insecticide intoxication.

*Respiration and respiratory quotients.*—The measurement of the rate of oxygen consumption and the respiratory quotients of intoxicated insects are other functions of the entire organism that may be conveniently observed and measured without the sacrifice of the test insect. Rather extensive investigations of the rate of oxygen consumption of insects intoxicated with a variety of insecticides have been reported by Harvey & Brown (33) and numerous other investigators (12, 41, 42, 56). With the exception of rotenone and the lethanes (33), the respiratory rate generally becomes accelerated in stage II (hyperactivity), appears to approach a maximum in the later period of stage II and in III (lack of co-ordination), then declines somewhat more slowly to normal and below normal levels in stage VI (apparent death). The generally prevalent concept is that the increased oxygen consumption is a direct manifestation of the excessive physical activity accompanying intoxication. In this connection, it is interesting to note the observations of Hassett (34) on the effects of ryanodine on the respiration and activities of *P. americana*. Although there is a gradual paralysis without the generally expected excessive physical activity, the rate of oxygen consumption increases up to seven times that of the control roaches that have been mechanically immobilized. A further difficulty in ascribing the enhanced rate of oxygen consumption to physical activity as a result of insecticidal intoxication is in Lord's (42) data revealing that *Tribolium castaneum* (Herbst) treated with abrasive dusts have an increased rate of oxygen consumption. This effect was most noticeable at low relative humidities when the treatment was with $Al_2O_3$. Vernberg & Meriney (74) also reported an increased oxygen uptake in the case of *Drosophila melanogaster* Meigen exposed to low relative humidities. Jochum (37) observed an increase in the rate of oxygen consumption of *L. decemlineata* intoxicated with parathion. Jochum has presented an interesting argument to explain the relations that exist between water losses and increased respiratory rate. He suggests that the increased respiratory rate is

a manifestation of the water loss rather than a fulfillment of the needs brought about by excessive physical activity.

The effects of insecticides on respiratory quotients have not been so extensively reported in the literature as the gross respiratory rates. Buck & Keister (12), investigating the effects of DDT on adult *P. regina*, reported R.Q.'s of 0.90 in controls and 0.96 in poisoned flies. These values suggested a largely carbohydrate fuel. In later work, these same authors (13) observed a change in R.Q.'s from 0.74 to 0.84 in DDT-poisoned larvae of this same species, leading to the same conclusion of increased carbohydrate utilization. Buck & Keister point out the similarity between respiration phenomena observed in intoxicated insects and insects experiencing environmental stresses such as flight, etc. The respiration and R.Q. experiments just discussed have, for the most part, been restricted to phenomena occurring over a period of several hours. Ludwig (43), in a study of the effects of DDT on *P. japonica* larvae, reported R.Q.'s of 0.7 to 0.8 during the first two days after treatment. The R.Q. was then reported to drop to 0.6 to 0.7, indicating a utilization of carbohydrates and fats in the initial period followed by a shift to utilization of fat reserves.

*Heart action.*—The insect heart, in certain species, can be observed without the sacrifice of the experimental animal. Although even minor disruptions of heart action in the higher animals usually become critical, this does not appear to hold true for insects (39). Krijgsman (40) has observed that the heart continues to beat after the cessation of somatic movement. The heart of American cockroaches was observed in rhythmic contraction after four hours of prostration from TEPP intoxication, despite the apparent lack of hemolymph in the body cavity (60). This continuation of heart action after the apparent cessation of other manifestations of muscular action is difficult to explain, unless a specific hormonal stimulation in connection with the intoxication phenomena is postulated. Extracts of the corpora cardiaca have been reported to increase the frequency and amplitude of heartbeat (see Table I). The dinitro compounds have been reported to stimulate heartbeat in the American cockroach while rotenone steadily depressed the heart (55).

## Neuroendocrine Function

The role of the neuroendocrine system of insects in controlling or regulating physiological activity is an increasingly complex picture and appears to be analogous to mammalian endocrine function in many respects. This subject, which has been reviewed by Van der Kloot (73), includes neurohormonal influence on the processes of moulting, diapause, activity rhythms, water metabolism, reproduction, nerve stimulus, muscle control, and, finally, nervous control of endocrine secretion. The direct or indirect action of the organic insecticides on nerve function could upset hormonal balance and thus indirectly disturb a variety of physiological and biochemical activities in the insect. The symptoms of insecticide intoxication might conceivably then be a response to hormone action, as well as to nerve function or the interaction of the two, as indicated in the previous section. Recent data on the release of

neuroactive agents or hormones into the blood of intoxicated or stimulated insects point up one aspect of the problem.

Sternburg & Kearns (67; see also 65) first demonstrated the presence of a naturally derived substance in the blood of DDT-poisoned American cockroaches that altered spontaneous activity of excised nerve cords and was toxic when injected into DDT-resistant house flies. A similar toxin was also correlated with pyrethrum poisoning in roaches and caused paralysis when injected into adult flesh flies, *Sarcophaga crassipalpus* Macquart (9). The source of the neurotoxin, which was reported by Sternburg & Kearns and is produced by either mild electrical shocks or DDT poisoning, has been definitely shown to be elaborated from the abdominal ventral nerve cord as the result of constant bombardment of the sensory central synapses by excessive afferent impulses generated in the sensory nerves (66). This neurosecretion was found to increase spontaneous activity in lower concentrations or up to early prostration but suppressed activity in late prostration when a high blood titer was reached (62).

TEPP, when applied to isolated nerve cords, caused the release of a neuroactive agent chromatographically identical to that produced by DDT. When this substance was released during high spontaneous nervous activity, it induced a subsequent blocking of activity. Washing with fresh TEPP in saline restored spontaneous activity, although the nerve cholinesterase was completely inhibited (64).

Colhoun (19, 20, 21) reported on the nerve cord-stimulating activity of blood from TEPP- or DDT-prostrate American cockroaches which contained high titers of corpora cardiaca and allata hormones, as well as unidentified substances. Extracts from the corpora cardiaca were found to excite isolated nerve cords and to simulate the effect of blood from intoxicated roaches on nerve and heart preparations (20). The corpora allata extract had no such effect.

Becht (7) has investigated the effect of lindane and DDT on the chordotonal sense receptors in the coxotrochanteral joint of the American cockroach. An injected dose of lindane, producing convulsions and paralysis, had no apparent effect on these sensory organs. On the other hand, DDT caused marked convulsions through the experimental period, and during the first hour the nerve fibers were in a state of high spontaneous excitation. This was later followed by a depression in activity in which nerve response was reduced to a few impulses. This increase and depression is suggestive of the neurohormone action on the central nerve cord, as reviewed previously, and the failure of lindane to induce this effect may point to an exception in the release of this factor in the blood.

Beament (4) observed that roach nymphs (*P. americana*) became paralyzed when subjected to mechanical immobilization, which induced violent struggling, or to electrical or mechanical stimulation; he reported that the paralyzing agent was carried on the blood.

Ozbas & Hodgson (57) determined that extracts of the corpora cardiaca from *P. americana* and *Blaberus craniifer* Burmeister decreased the frequency of spontaneous nerve impulses, although a few occurrences of slight stimula-

tion involving low dosages occurred. Later work demonstrated that neurosecretory material stored in the corpora cardiaca was depleted by electrical shocks and forced hyperactivity. It was suggested that the release of neurosecretory substances from the corpora cardiaca may be a response to stress situations (36). The apparent contradictory findings that the corpora cardiaca extracts may either excite or depress spontaneous nerve activity has been resolved by Milburn *et al.* (50) and is similar to the action of the neuroactive agent released by the ventral nerve cord. Milburn *et al.* observed that the corpora cardiaca extracts temporarily increased activity of the ventral nerve cord but that high concentrations occasionally depressed or blocked. The DDT toxin of Sternburg & Kearns was shown not to be the same as the active principle of the corpora cardiaca extracts. The corpora cardiaca extracts appeared to exert their excitory effects by suppressing the inhibiting action of the subesophageal ganglion and thereby increasing efferent nerve activity.

Heslop & Ray (35) have demonstrated that the ability of American cockroaches (*P. americana*) to tolerate stress in the form of prolonged mechanical agitation can be correlated directly to their degree of resistance to DDT. It was postulated that a generalized stress syndrome is induced in addition to the specific response to the insecticide. The symptoms of physical stress and DDT poisoning, such as increased oxygen consumption followed by a fall in respiration and a depletion in high-energy phosphate compounds, were similar.

From the previously mentioned results, it is apparent that insecticide intoxication produces secondary effects in the form of releases of abnormal amounts of neurophysiologically active substances. Similar results are obtained by the application of non-specific stress to the organism. The origin of the active factor is, in part, from the ventral nerve cord and, possibly, the corpora cardiaca. Low concentrations of these substances excite nerve activity, whereas higher concentrations depress or block activity, and, in the case of ventral nerve cord factor, the low and high concentrations may be correlated to the hyperactive and prostrate stages of DDT intoxication, respectively. Further, blood-borne agents originating from insecticidal or non-specific stress are capable of inducing paralysis and death of the insect.

## Intermediary Metabolism

*High-energy phosphates.*—Stress and DDT intoxication have other symptoms in common, such as increased respiration and depletion of high-energy phosphates under certain conditions (6, 23, 35, 75). Winteringham (75), however, observed no significant ATP loss in flies starved to the point of prostration. Injection of DDT-treated *Galleria* larvae with high doses of ATP protected the larvae to a limited extent from symptoms and death, but results were not consistent. Thus, it was stated that reduction of ATP was not the sole cause of prostration in this insect (6).

The effect of DDT and its analogues on the ATP-$P^{32}$ exchange reaction catalyzed by mosquito sarcosomes was investigated by Gonda *et al.* (31). All compounds tested inhibited the reaction by nearly the same extent, and

no correlation was found between toxicity to mosquito larvae, *Aedes aegypti* (Linnaeus), and their effect *in vitro* on the exchange reaction.

*Carbohydrate utilization.*—Other effects induced by DDT poisoning that are suggestive of stress are the depletion of carbohydrate reserves, the loss of body weight, and a general increase in blood amino nitrogen content (similar to changes that have been observed in starved insects) (23, 36, 43, 46).

Ludwig (43) indicated that DDT-treated Japanese beetle larvae (*P. japonica*) die from exhaustion of carbohydrate reserves as a consequence of increased muscular activity. DDT-intoxicated American roaches lost about 90 per cent of their glycogen and glucose, but this did not appear to be the ultimate lethal action of the insecticide, since insects still succumbed when protected from carbohydrate loss by continuous anesthesia or administration of glucose (49). Buck & Keister (12) also substantiated this latter view when they demonstrated that there was more respirable substrate left in DDT-killed blow flies (*P. regina*) than in the starved controls. However, Beard (6) has demonstrated with *G. mellonella* larvae, paralyzed by the venom of *Microbracon hebetor* (Say), that the lethal action of DDT was suspended as long as muscle action remained blocked. When muscle activity was resumed after one week, typical symptoms and death occurred among the DDT-treated larvae, while the paralyzed controls pupated normally. In contrast, non-paralyzed DDT-treated larvae died after a much shorter interval.

*Oxidative metabolism.*—The effects of DDT on insect aerobic carbohydrate metabolism (2, 3, 11, 28, 44, 45, 61) and oxidative phosphorylation (44, 72) have been studied with little conclusive evidence forthcoming as to the actual part these play in the lethal action of the insecticide.

Recent work by Barsa & Ludwig (3) indicates that DDT ($10^{-3}M$) has a specific effect on the cyanide sensitive respiration, in that it inhibits only cytochrome oxidase and succinic dehydrogenase, but not the alcohol, glucose, lactic, α-glycerophosphate, or isocitric dehydrogenases, in the homogenates of the yellow mealworm (*Tenebrio molitor* Linnaeus) and house fly. However, the fact that the non-toxic metabolite DDE [1,1,dichloro-2,2-bis(*p*-chlorophenyl)ethylene] and other DDT-related compounds also inhibited the enzymes of the succinoxidase system in house fly homogenates led Anderson *et al.* (2) to conclude that this inhibition was not a prime factor in the mode of action of DDT. Fukami (28) has presented evidence that DDT is not an *in vivo* inhibitor of the respiratory enzyme systems in *P. americana* at the time of paralysis. Similarly, O'Brien (53, 54) has concluded that malathion inhibition of the succinoxidase and pyruvate oxidation systems is not important in its insecticidal action.

Fukami demonstrated the *in vivo* inhibition of the succinoxidase (26, 27) and L-glutamic dehydrogenase (29, 30) systems of the American cockroach injected with rotenone, and he concluded that the primary action of rotenone was the inhibition of respiratory metabolism in the nerve and muscle of insects (28). The *in vivo* toxicity of rotenone and its derivatives were comparable to the degree of glutamic dehydrogenase inhibition by each of them (30). Rotenone interfered both *in vivo* and *in vitro* with oxidative phosphorylation in locusts, *Locusta migratoria* (Linnaeus), but this effect was thought to be

produced indirectly by the inhibition of the respiratory system and not by the uncoupling of oxidative phosphorylation (72).

*Amino acid metabolism.*—The biochemical importance of the free amino acids in insect blood is largely unknown, although certain of them may serve as readily available substrate sources for tricarboxylic acid cycle oxidation (76). There is a high degree of variation of blood amino acid content among insect species and at different stages of growth, as reviewed by Florkin (25). Alanine, arginine, histidine, lysine, proline, valine, leucine, and, in some cases, glutamic acid and glutamine are found in highest concentration. Environmental factors, such as temperature, starvation, and insecticide intoxication, influence blood levels of the free amino acids.

Florkin (25), working with the diapausing pupae of several moth species, has observed that alanine increases in the hemolymph and total glutamic acid falls at low temperature. A decrease was also noted for histidine, lysine, and proline.

Joseph (38), in analyzing the blood of *T. molitor* treated with DDT, found large increases in amino nitrogen, reducing substances, and non-protein nitrogen but no change in protein nitrogen. Amino acids showing the largest increases were alanine, glutamic acid, proline, and histidine. The author further compared these findings with similar conditions induced by starvation and has suggested starvation following DDT poisoning as the cause of death. Winteringham (76) reported significant increases in free glutamine in house flies treated topically with di-isopropylphosphoroforidate and with DDT. Proline was observed to disappear from the blood of DDT-treated *P. americana* exhibiting symptoms, and there was corresponding increase in glutamine (22). When the symptoms were alleviated by exposing the insects to increased temperature, proline reappeared in the blood.

In contrast to the work of Joseph, the concentration of most other amino acids remained unchanged. Pyrethrins and lindane caused only slight decreases in proline, while dieldrin and TEPP produced no changes. It was concluded that the disappearance of proline during poisoning is directly linked to the increased utilization of glutamic acid for the synthesis of glutamine and *alpha*-ketoglutarate. A possible explanation for these fluctuations may be found in the elevated rate of catabolism and the expected establishment of a negative nitrogen balance in the poisoned or starved insect.

Since alanine, glutamate, and aspartate can arise directly from intermediates of carbohydrate metabolism, their increase in the blood may be linked to the depletion of carbohydrate reserves. The transamination reactions have been demonstrated in a number of insect species, as reviewed by Bheemeswar (8), and are as follows:

$$\text{glutamate} + \text{pyruvate} \rightleftarrows \textit{alpha}\text{-ketoglutarate} + \text{alanine}$$

$$\textit{alpha}\text{-ketoglutarate} + \text{aspartate} \rightleftarrows \text{glutamate} + \text{oxaloacetate}$$

McAllan & Brown (47) were able to induce tremors, paralysis, and even death when they injected glutamate and aspartate in the hemocoel of American cockroaches, but similar doses of the corresponding *alpha*-keto acids had no observable effect. DDT, toxaphene, endrin, and malathion were found to

no correlation was found between toxicity to mosquito larvae, *Aedes aegypti* (Linnaeus), and their effect *in vitro* on the exchange reaction.

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*Oxidative metabolism.*—The effects of DDT on insect aerobic carbohydrate metabolism (2, 3, 11, 28, 44, 45, 61) and oxidative phosphorylation (44, 72) have been studied with little conclusive evidence forthcoming as to the actual part these play in the lethal action of the insecticide.

Recent work by Barsa & Ludwig (3) indicates that DDT ($10^{-3}M$) has a specific effect on the cyanide sensitive respiration, in that it inhibits only cytochrome oxidase and succinic dehydrogenase, but not the alcohol, glucose, lactic, α-glycerophosphate, or isocitric dehydrogenases, in the homogenates of the yellow mealworm (*Tenebrio molitor* Linnaeus) and house fly. However, the fact that the non-toxic metabolite DDE [1,1,dichloro-2,2-bis(*p*-chlorophenyl)ethylene] and other DDT-related compounds also inhibited the enzymes of the succinoxidase system in house fly homogenates led Anderson *et al.* (2) to conclude that this inhibition was not a prime factor in the mode of action of DDT. Fukami (28) has presented evidence that DDT is not an *in vivo* inhibitor of the respiratory enzyme systems in *P. americana* at the time of paralysis. Similarly, O'Brien (53, 54) has concluded that malathion inhibition of the succinoxidase and pyruvate oxidation systems is not important in its insecticidal action.

Fukami demonstrated the *in vivo* inhibition of the succinoxidase (26, 27) and L-glutamic dehydrogenase (29, 30) systems of the American cockroach injected with rotenone, and he concluded that the primary action of rotenone was the inhibition of respiratory metabolism in the nerve and muscle of insects (28). The *in vivo* toxicity of rotenone and its derivatives were comparable to the degree of glutamic dehydrogenase inhibition by each of them (30). Rotenone interfered both *in vivo* and *in vitro* with oxidative phosphorylation in locusts, *Locusta migratoria* (Linnaeus), but this effect was thought to be

produced indirectly by the inhibition of the respiratory system and not by the uncoupling of oxidative phosphorylation (72).

*Amino acid metabolism.*—The biochemical importance of the free amino acids in insect blood is largely unknown, although certain of them may serve as readily available substrate sources for tricarboxylic acid cycle oxidation (76). There is a high degree of variation of blood amino acid content among insect species and at different stages of growth, as reviewed by Florkin (25). Alanine, arginine, histidine, lysine, proline, valine, leucine, and, in some cases, glutamic acid and glutamine are found in highest concentration. Environmental factors, such as temperature, starvation, and insecticide intoxication, influence blood levels of the free amino acids.

Florkin (25), working with the diapausing pupae of several moth species, has observed that alanine increases in the hemolymph and total glutamic acid falls at low temperature. A decrease was also noted for histidine, lysine, and proline.

Joseph (38), in analyzing the blood of *T. molitor* treated with DDT, found large increases in amino nitrogen, reducing substances, and non-protein nitrogen but no change in protein nitrogen. Amino acids showing the largest increases were alanine, glutamic acid, proline, and histidine. The author further compared these findings with similar conditions induced by starvation and has suggested starvation following DDT poisoning as the cause of death. Winteringham (76) reported significant increases in free glutamine in house flies treated topically with di-isopropylphosphoroforidate and with DDT. Proline was observed to disappear from the blood of DDT-treated *P. americana* exhibiting symptoms, and there was corresponding increase in glutamine (22). When the symptoms were alleviated by exposing the insects to increased temperature, proline reappeared in the blood.

In contrast to the work of Joseph, the concentration of most other amino acids remained unchanged. Pyrethrins and lindane caused only slight decreases in proline, while dieldrin and TEPP produced no changes. It was concluded that the disappearance of proline during poisoning is directly linked to the increased utilization of glutamic acid for the synthesis of glutamine and *alpha*-ketoglutarate. A possible explanation for these fluctuations may be found in the elevated rate of catabolism and the expected establishment of a negative nitrogen balance in the poisoned or starved insect.

Since alanine, glutamate, and aspartate can arise directly from intermediates of carbohydrate metabolism, their increase in the blood may be linked to the depletion of carbohydrate reserves. The transamination reactions have been demonstrated in a number of insect species, as reviewed by Bheemeswar (8), and are as follows:

$$\text{glutamate} + \text{pyruvate} \rightleftarrows \textit{alpha}\text{-ketoglutarate} + \text{alanine}$$

$$\textit{alpha}\text{-ketoglutarate} + \text{aspartate} \rightleftarrows \text{glutamate} + \text{oxaloacetate}$$

McAllan & Brown (47) were able to induce tremors, paralysis, and even death when they injected glutamate and aspartate in the hemocoel of American cockroaches, but similar doses of the corresponding *alpha*-keto acids had no observable effect. DDT, toxaphene, endrin, and malathion were found to

inhibit transamination *in vitro* but not under *in vivo* conditions; therefore, inhibition was considered an artifact attributable to enzyme absorption by the insecticide suspension. From this work emerges the possibility of amino acid toxicity to insects and the speculation as to what contribution our knowledge of it may make in the total summation of insecticide intoxication.

*Excretion.*—Patton *et al.* (59) have observed that the cyclodiene insecticides have a specific inhibiting effect on malpighian tubule excretion. The ability of the tubes of *P. americana* to excrete was correlated with their ability to reduce blue tetrazolium, indicating that the system is energized by oxidative enzymes. Endrin almost completely limited excretory activity and tetrazolium reduction by the tubes, and the next most effective, in descending order, were isodrin, dieldrin, heptachlor, aldrin, and chlordane. DDT and parathion were without effect.

## Discussion

Investigations into the mechanisms of insecticide action are revealing an increasingly complex picture in which the disturbance of perhaps one physiological system by a toxicant will, in turn, unbalance other closely integrated systems, until the sequence of events resulting in the death of the insect is exceedingly difficult to analyze. A case in point appears to be the nervous and hormonal interaction in controlling physiological activity, and the opposing actions of different hormones suggest a delicately balanced control system analogous to that found in mammals. The excitation of the nervous system by an insecticide or by other stress factors appears to initiate a chain of events beginning with the release of neuroendocrine secretions which, in turn, induce responses in target organs. The continued secretion of abnormally large amounts of these substances may be sufficient to eventually result in physiological changes great enough to cause paralysis and death of the insect. Many of the symptoms of insecticide poisoning appear to be very similar to those produced by neuroendocrine material; this again suggests a relationship between the two.

Insecticides appear to produce symptoms and mortality by both primary or specific action and by secondary or non-specific action. There is good evidence for the former in nearly all groups of the organic insecticides, as indicated by unique symptoms and biochemical changes.

The inhibition of cholinesterase by the organophosphorus compounds has been fairly well established (78). Rotenone appears to be an *in vivo* inhibitor of L-glutamic dehydrogenase and succinoxidase systems. The cyclodiene insecticides inhibit the excretory processes of the Malpighian tubules and elicit characteristic symptoms of intoxication. The importance of the specific action is more apparent if lethal time is relatively short.

Secondary or non-specific effects appear to be characterized by a common syndrome of symptoms induced by either insecticidal or non-specific stress. When the period of lethal action is extended by either the characteristics of the insecticide or the natural tolerance of the species, the secondary effects become more manifest. Tolerance to DDT appears to be correlated with tolerance to mechanical stress (35), and, conversely, protection of poisoned

insects from prolonged muscular activity by venom paralysis or anesthesia seems to confer upon the insect a certain degree of immunity to the insecticide (5, 49). Symptoms that appear to be secondary in nature are loss of body weight, depletion of carbohydrate stores and, possibly, high-energy phosphates, increase in oxygen consumption, loss of body water or an abnormal redistribution caused by a dysfunction in water regulation, muscular spasms, and, finally, an abnormal release of neurosecretions into the hemolymph, which may induce any of the above symptoms.

Again, it is relatively difficult to analyze the importance of the primary and secondary effects in contributing to lethal action or even clearly distinguish between the two. This difficulty is further compounded by the interaction of the stresses, other than those attributable to intoxication, that may be inadvertently and irregularly supplied as a part of the experimental routine.

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# ECOLOGICAL ASPECTS OF PLANT VIRUS TRANSMISSIONS[1,2,3]

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The movement of insects from one host plant to another is the commonplace activity upon which all transmission of plant virus diseases by arthropods depends. This movement is conditioned by many factors, the normal life-history of the insect, its host range and host preferences, the availability and condition of these hosts, and their status as virus reservoirs. Superimposing their determinative influence on all these biotic factors are the physical factors of the environment. This discussion is concerned with the interrelationships of these factors.

## Movement, Dispersal, and Migration

Movement, dispersal, and migration are terms, the definition of which varies with the user. Movement should perhaps be restricted to changes of position intramurally; that is, an apterous aphid moving from one part of the plant to another or from plant to plant in the same location would be considered in movement. Alate aphids rising above a potato field and settling down again would fall in the same category.

Dispersal has been defined as flights within natural breeding areas [Linn (84); Severin (102)], and migration as flights out of the natural breeding areas. Perhaps "permanent" would be a better word than "natural." Dispersal would include such phenomena as flight of aphids from a primary to a secondary host; e.g., the short flights of *Piesma quadrata* Fieber from the woodlands, where it overwinters, to young sugar beets, and the back-and-forth movements from weeds to cultivated crops of many vector species. Dispersal is no doubt conditioned by many instinctive reflexes and the local pressures of the environment, but it still involves considerable choice, *in situ*, and in host-plant selection.

Migration, the flight out of permanent breeding grounds, usually involves long distances, sometimes hundreds of miles, and infers an obligatory movement imposed by climate or other factors. Dispersal could be a prelude to migration because, at that time, the insect, moving in short flights, is vulnerable to strong convection currents and high winds. Within the limits im-

[1] The survey of the literature pertaining to this review was concluded in December, 1959.

[2] Published with the approval of the Director as Technical Paper No. 267 of the Pineapple Research Institute of Hawaii.

[3] This review should be read in conjunction with that of Broadbent, L., and Martini, C., *Advances in Virus Research*, 6, 93–135 (1959).

posed by season, dispersal of a migrating insect can occur at the end of the migration when the insect is occupying its temporary breeding grounds.

Simpson (118) restricted the use of the term "migration" to movements in the spring and fall between primary and secondary hosts and "dispersal" to movements occurring within or between plantings of secondary host plants.

Migration, according to Davidson (31), has a specialized meaning with respect to aphids, namely, the flight of sexual and asexual forms between winter and summer hosts. Aphids are called "migratory" or "non-migratory" according to whether or not they so alternate. Johnson's usage (73) differs somewhat in that he includes some aphid flights as migrations where there is no alternation of summer and winter hosts.

Lawson *et al.* (82) preferred the word "dissemination" to "dispersal" or "migration"; the objection to the word "dispersal" was that it, by definition, connotes no directional movement and to "migration" because it has certain specialized (subjective) meanings when used in connection with birds, mammals, and fishes. Neither objection seems valid; in fact, Lawson *et al.* found it convenient to use the term "flight" to describe movement from one place to another. It is not at all certain that the "migration" stimuli of insects differ fundamentally from those of any other animals, whether it be crowding, food supply, or just the urge to get up and go, which is associated with maturation.

A distinction between "active" and "passive" migration has been made; "active" migration is held to be purposeful flight, taken only in still, dry air for short distances, while "passive" migration has been taken to involve long distance flights [Doncaster (41)]. Whether "passive" flights are in fact flights, and not merely involuntary wind-controlled movement of buoyant particles, would be difficult, if not impossible, to determine.

*Methods of measuring vector populations and their movements.*—Dispersal and migration are quantitative and, with the exception of the sugar-beet leafhopper, have been studied most in connection with aphid vectors.

Much of the earlier work was done by Davies (33), who periodically examined 100 leaves, taken at random, from varying positions on the plants during the season. Initial infestation and its growth and movement throughout the crop could therefore be estimated. Any sampling method is suspect, but any such method consistently followed will provide comparable data. For tracing the movement of aphids within fields, an extension of the counting method was necessary. Davies developed a method for locating the aphids on the sampled leaves, and weekly records disclosed that at least 84 per cent of each of two species moved from leaf to leaf during the weekly period.

To avoid variation in size and position of leaves, some workers have attempted estimates of aphids per plant [Bald *et al.* (5)]. When the leaf position is considered, the sampling method becomes complicated; Bald *et al.* (6), however, took the total count per plant and then divided the lower

leaves into two groups: the basal leaves, which are the lowest two or three, and the "ground canopy," consisting of the next three leaves, which form a canopy over the ground without touching it. Location of nymphs differed with species, and adults were more randomly distributed than nymphs. Broadbent (13) estimated aphid density by multiplying the number of aphids on upper, middle, and lower leaf by the average number of leaves for each zone.

Shands *et al.* (105) counted wingless aphids by using two subunits: one consisted of the terminal and two opposite basal leaflets from three leaves that were taken from the top, middle, and bottom of the plant, and the other, one half of each of those leaflets. The effect of using the second was to greatly reduce labor, and the results were comparable.

Strickland (127) developed an aphid-counting grid after testing volumetric methods. The latter method was shown to be inadequate when it was found that 2500 small nymphs or 600 third-instar nymphs occupied the same volume (1 cc.) as did 250 alatae. Combinations of the three-leaf method and the grid count were used by Church & Strickland (23) in sampling aphids on Brussels sprouts.

The standard unit for determining populations of sugar-beet leafhoppers on wild and cultivated hosts has been, for many years, 50 sweeps of a net [Carter (21); Fox (50)]. The method has obvious disadvantages [DeLong (37); Gray & Treloar (55)] but has universal applicability within its limits. It is practically useless to determine the early movements of leafhoppers into beet fields when the plants are very small. The appearance of males is the best criterion of the spring movement [Douglass *et al.* (45)], and the first quantitative counts are made by the "hand-and-knee method," wherein the observer moves on hands and knees along a row of sugar beets, gently disturbing the plants as he moves along and making an actual count of the leafhoppers disturbed. Hills (64) developed a sampling cage, one foot square, which gives a somewhat more accurate count, and Shands *et al.* (110, 112) have described wind-vane aphid traps.

Survey methods in the United States have been brought to a fair degree of standardization and have been published by the Plant Pest Control Branch of the Agricultural Research Service (3). Vectors included in these are the beet leafhopper and the major vector species of aphids. The onion thrips is also included, but on onions only, not on any virus host. Some survey methods are necessarily drastic. Strickland (126), in order to determine the distribution and density of mealybug populations on cacao trees, felled 12 trees on each of 10 one-acre plots each month and counted all the mealybugs found on trunk, branches, twigs, and leaf canopy.

Many methods have been devised for estimating the numbers of insects in flight. For aphids the sticky trap has been widely used [Broadbent *et al.* (16); Doncaster & Gregory (42)]; for leafhoppers, Lawson *et al.* (82) have reviewed the use of various mechanical devices that depend on air movement for their efficiency. For sampling air-borne mite populations, Staples &

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Survey methods in the United States have been brought to a fair degree of standardization and have been published by the Plant Pest Control Branch of the Agricultural Research Service (3). Vectors included in these are the beet leafhopper and the major vector species of aphids. The onion thrips is also included, but on onions only, not on any virus host. Some survey methods are necessarily drastic. Strickland (126), in order to determine the distribution and density of mealybug populations on cacao trees, felled 12 trees on each of 10 one-acre plots each month and counted all the mealybugs found on trunk, branches, twigs, and leaf canopy.

Many methods have been devised for estimating the numbers of insects in flight. For aphids the sticky trap has been widely used [Broadbent *et al.* (16); Doncaster & Gregory (42)]; for leafhoppers, Lawson *et al.* (82) have reviewed the use of various mechanical devices that depend on air movement for their efficiency. For sampling air-borne mite populations, Staples &

Allington (121) used grease-coated 1×3 in. slides and concluded that good estimates of mite populations in volunteer wheat could be obtained by use of a single trap. The whiteflies have, thus far, eluded any attempt to determine their movements on a quantitative basis, as have the mandibulate vectors, beetles and grasshoppers.

The height of insect vector flights has received attention. Broadbent (9) found that 68 per cent of *Myzus persicae* (Sulzer) were taken at a mean height of 68 in., 25 to 28 per cent at 38 in., and 5 to 8 per cent at 7 in. By means of sticky drum traps, Nowak (88) caught this same species up to 45 meters, but Glick (54), reporting on the results of airplane trapping, recorded *M. persicae* taken at 3000 feet and *Macrosiphum pisi* (Harris) at 200, 1000, 2000, and 4000 feet.

Johnson & Penman (74) found a linear relationship between the logarithm of aphid density and the logarithm of height with a range of 10 to 2000 feet. Shands *et al.* (113) studied the low elevation movement of aphids for several years, using a combination of wind-vane traps and tall tanglefoot screens. The three species of aphids studied, buckthorn, green peach, and potato aphids, all had their peak populations around the 25 foot level. Heathcote (58) used yellow water traps [after Moericke did so in 1951 (86a)] and yellow cylindrical traps [after Broadbent *et al.* (16)] to determine flight at different "heights," that is, from ground level to 152 cm. These studies obviously pertain to movement and dispersal; a migrating insect would not get very far travelling six feet from the ground. Dickson *et al.* (40) found that a five-foot high trap gave the best relative count. Traps at one, five, and 12 feet revealed differences between species, *Aphis spiraecola* Patch showing somewhat higher counts at the higher level, while the reverse was true of *Aphis gossypii* Glover. In all cases, more aphids were caught on the leeward side of the citrus trees than on the windward side.

Broadbent (9) found a relationship between height of trap and species caught, different species flying at different heights; the highest trap was five to six feet from the ground, and voluntary flight decreased with increasing height. Perhaps both movement and dispersal are primarily voluntary, and, the closer to the ground the aphid flies, the less the possibility of its being swept into obligatory migration. Leafhoppers have been trapped in various types of mechanical devices [Lawson *et al.* (82)] at heights up to 127 feet [Hills (65)].

Migration is not necessarily achieved in a single flight. Dorst & Davis (43) traced long distance movements of the beet leafhopper by planting a series of 100 square foot plots of favored weed hosts in the desert areas over which the insect migrates. The nearest winter breeding area to the sugar-beet fields is about 200 miles distance. Daily collections on these plots definitely placed the origin as well as the time and magnitude of dispersal of leafhoppers. Douglass *et al.* (45) determined the time and magnitude of dispersal of leafhoppers into beet fields by the use of Hills' sampler [Hills (64)], which traps the insects in circular areas containing one square foot.

Strickland caught a few small nymphs of mealybugs on sticky traps in the cocoa-growing area of Ghana. The numbers were not as significant as the indication that wind could be a factor in the movement of the insects (124).

### Factors Affecting Movement of Vectors[4]

Factors affecting movement of vectors are a complex of biotic and physical factors. For the aphids, Davies (35) demonstrated, with controlled laboratory experiments, that high humidities inhibited flight of *M. persicae*, and that temperatures from 70° to 90°F. were the most favorable. Thomas & Vevai (129) extended these studies and suggested some arbitrary standards for the flight of aphids in general. These were: (*a*) a temperature above 70°F.; (*b*) a relative humidity below 80; (*c*) a wind velocity below 5 m.p.h.; and (*d*) a big difference in maximum and minimum temperatures (more than 12°F.) for the day. The most important factor limiting flight appeared to be wind velocity. Davies' laboratory experiments were thus confirmed in general by the field data of Thomas & Vevai, but later experiments under controlled conditions by Broadbent (11) indicated that reactions to relative humidity are complex.

Increases in relative humidity retarded flight activity; changes to a lower level increased activity, but aphids adjusted to humidities between 50 and 80 per cent and flew readily at these humidities with temperatures below 80°F. High humidity and high temperature (90°F.) sometimes inhibited flight. Light intensity between 100 and 1000 ft. c. made little difference to flight, but below 100 ft. c. flight activity declined rapidly. These light intensities were from artificial light. Markkula (85) recorded differences in aphid take-off in varying degrees of sunlight. Forty-three, 20, and 11 take-offs per minute of *Brevicoryne brassicae* (Linnaeus) were observed with full sunshine, thin clouds, and dense clouds, respectively.

If all the possible physical factors in the micro-environment are considered—the varying humidities, temperatures, and light exposures that could be found within a few feet—generalizations based on broad field studies might be modified, but only in a very limited degree.

Leafhopper movements have been studied extensively. The physical factors most closely associated are those of temperature and wind. Lawson *et al.* (82) found that *Circulifer tenellus* (Baker) flew when temperatures were above 60°–64°F. and especially in the crepuscular periods near sunrise and sunset. Since temperatures are more likely to be favorably above the threshold in the evening, flights at that time are more common. The number of leafhoppers moving on any particular day is proportional to the temperature. The sexes react differently to winds of low velocity, the males tending to swarm around conspicuous objects. With winds of higher velocity, both sexes are carried along in the wind stream, not necessarily the prevailing wind, but in those that are blowing at the time when light and temperature

[4] Movement in the inclusive sense.

conditions are most favorable for flight. A low correlation between catch of leafhoppers and humidity was shown to be actually attributable to the fact that both catch and humidity were correlated with temperature. Lawson *et al.* believed that most of the flying population travelled below 10,000 feet on "surface" winds; in fact, the bulk of their catches were from five to 50 feet, with only a few caught at the 130 foot level. Ball (7), however, reported finding beet leafhoppers on the top of Pike's Peak at 14,000 feet, and, while flights at this altitude are probably rare, they would account for long-distance migrations of the insect into areas far east of the permanent breeding grounds. Douglass (44) summarized the records of the occurrence of curly-top in these areas, and, since then, there have been additional records from Maryland in 1958 and 1959 [Schneider (101); Heggestad & Moore (60)]. It is perhaps significant that these last records are for the virus disease, not the leafhopper. Douglass quotes Glick's (54) findings on the influence of air currents on insect migration. The rougher the air, the greater the proportion of insects found at the higher elevations. Leafhoppers at active temperatures live only a short time if unfed. This would require that insects in obligatory long-distance migration be carried passively in air currents at temperatures lower than their threshold of development, as was suggested by Severin (102) and Carter (19).

The beet leafhopper weed-host complex, according to Douglass (44), has changed in favor of the insect in some areas during the last 30 years, but, unless the insect is becoming acclimatized, as Douglass suggests, the end point of the long-distance migration will be in an area climatically unsuitable for the insect's survival [Carter (21)]. Even the permanence of an extension of the breeding grounds north and east into the southern Great Plains is dependent on subnormal rainfall [Douglass *et al.* (46)].

The leafhopper transmitting aster yellows virus, *Macrosteles fascifrons* (Stål), has not been considered a long-distance migrant, but recent data indicate that this insect migrates into Canada from the northern United States. Extensive damage to lettuce and other susceptible crops occurs in Manitoba and, only slightly less, in Saskatchewan. Fourteen per cent of the immigrants were infective on arrival. Evidence of the extension of the migration as far north as Edmonton has been recorded [Sackston (100); Westdal *et al.* (136)].

### Host Plant Sequence the *Sine Qua Non* of Virus Transmission

It is conceivable that a monophytophagous insect, living on a host plant with a seed-transmitted virus, could exist, but only if the host and virus had reached a mutually tolerant equilibrium. Otherwise, the result would be the elimination of all three. All insect vectors of virus diseases, as far as is known, are polyphytophagous. Their movement between plants of the same host species can be conditioned by the insect's behaviour or the activities of man, while their movement between host plants of different species can be brought about by obligate facets of the insect's biology or by all the interrelated factors of their host-plant ecology.

Intrahost movement has been studied in connection with aphid biology. Davies (33) found that 84 per cent of *Macrosiphum euphorbiae* (Thomas) and 73 per cent of *Myzus persicae* changed site within 24 hours. There is considerable movement from leaf to leaf of adjoining plants when these are in contact [Czerwinski (29); Doncaster & Gregory (42)]. There is a difference between species. *M. persicae* is generally considered to be restless, particularly when temperatures are high. The apterae of *M. euphorbiae* move more freely from plant to plant than either *M. persicae* or *Aphis rhamni* (Fonscolombe) [Broadbent (13)]. Short, frequent flights are common on calm, warm evenings, or on calm, cloudy days, or in the early morning, and such flights will invariably result in alighting on other plants [Broadbent (11)].

Cornwell (27) studied the behavior of the mealybug vectors of swollen shoot disease on cacao. When diseased trees are cut down and slash piled, the mealybug populations are usually low, since many have left the infected tree early in the infection stage. The cut branches wilt, but wilting is retarded in the pile and many of the mealybugs remain on the cut wood, continuing to reproduce for nine weeks or more. Nymphs from this reproduction migrate, but when starved their movement is greatly retarded and they will die within a week.

The insect's biology may impose an obligatory change of host plant, and the prime examples of this are to be found among the aphids. Kring (78) states that true migratory (dispersing) aphids have three characteristics in common: (*a*) eggs are produced only on a few plant species, the primary hosts, and only viviparous females occur on other host plants, the secondary hosts; (*b*) primary host plants usually are members of older plant families than the secondary hosts; and (*c*) male aphids are produced only on secondary hosts, while the oviparous females are produced only on the primary hosts. According to this definition, *A. gossypii* is not a true "migrant" because both males and females are produced, but there is a formidable list of viruses of which it is a vector.

The primary hosts of *M. persicae*, the most ubiquitous known vector of viruses, vary with geographical location and climate. In Maine, wild plum is the only recognized primary host, and the aphid hibernates on this host in the egg stage [Simpson & Shands (119)]. In Holland, the insect may overwinter in glasshouses or in mangold clamps, but this is rare compared with hibernation on *Prunus serotina* Ehrhart, the American bird cherry, which has been planted in vast numbers as a protection for young coniferous forests, or on peach trees. According to Hille Ris Lambers (63), 97 per cent of migrants from winter hosts to summer hosts come from peach or *P. serotina*. In the northeast of Scotland, the insect overwinters mainly as apterous viviparae on various Cruciferae (savoy cabbages, cabbage, and Brussels sprouts) [Shaw (116)], and this is true also in England, particularly near towns and villages where many other cultivated crops also serve as overwintering hosts. In some parts of Europe, peach orchards are the main source of this aphid, which overwinters there in the egg stage, the winters being too severe for the viviparae to survive [Broadbent (13)].

The generalized seasonal history of aphids with alternating hosts is as follows: The eggs hatch in early spring, and most of the winged migrant forms mature in the third generation, moving to summer hosts, which are potatoes, and to many weed species. Reproduction continues on the secondary hosts until the end of the summer, when winged fall migrants fly to the primary hosts and initate colonies.

Since the magnitude of both spring flights outward from the primary hosts and the fall return flights depend on the production of alatae, the factors affecting this production are of some interest. Lal (80) related production of alatae to the decrease in the water content of the aphids, which was brought about by combinations of temperature and relative humidity, which also diminished the water content of their host plants. Crowding is associated with production of alatae, but this is probably caused by inadequacy of the water supply. Dry atmospheric conditions, a reduced water content in the host plants, and crowding of the aphids will be followed by an increase in the production of winged migrants. Kennedy *et al.* (77) confirmed the factor of water strain in the host plant in inducing increased production of alatae. The problem is complex. Sometimes a change of host plant will induce alatae production in the absence of water stress. *A. gossypii* growing on *Commelina nudiflora* Linnaeus in cages produced no winged forms, but, when *Bidens pilosa* Linnaeus grew as a weed in the cages, alatae developed profusely. Host transfer can be forced by the maturing of a weed host, either according to normal seasonal development or through drought.

Shands & Simpson reported on the production of alatae under greenhouse conditions (111). In this case, water stress was not a factor. They concluded that winged-form production was influenced by the size of the plant and the size of the aphid population.

Most authors agree that production of alatae is influenced by the factors of light, temperature, crowding, starvation, wilting of the host plant, and even parentage. Davidson (32) reported that winged migrants of *Aphis rumicis* (Linnaeus) developed as a result of an inherent tendency but that the actual number at any particular time in the development of the colony was influenced by overcrowding and the condition of the host plant.

It seems clear that the production of alatae in aphids, so essential to the spread of plant viruses, is brought about by this inherent tendency, essential to the survival of the species, and conditioned by physical and biotic factors of the environment.

Kennedy *et al.* (77) explained the effect of water shortage in the host as an effect of concentration of solutes in the sap, increasing at the expense of quantity available. This increased sap concentration increased the production of winged forms. A somewhat analogous situation is found in the case of *C. tenellus*. Although there is an inherent tendency to migrate coincident with maturation, the major factor is the condition of the host plants related to sap concentration [Carter (20); Lawson *et al.* (82)]. Thomas & Vevai (129) listed sunny weather, low relative humidity, large fluctuations in tempera-

ture, and a long dry period resulting in starvation as factors in the production of alatae. According to Wadley (132), temperatures are critical in the case of *Toxoptera graminum* (Rondani).

Interhost movement is often forced on the insect by the activities of man. As long ago as 1928, Wolcott (138) related the spread of sugar cane mosaic to the practice of weeding out small grass species among the cane plants. This resulted in the alatae flying off, but the apterae had no alternative but to move on to the nearest cane plant. Martin (86) reported the same sequence of events in Hawaii.

*Emilia sonchifolia* Linnaeus is a common weed in old pineapple fields and is frequently infected with tomato spotted wilt virus and heavily infested with *Thrips tabaci* Lindeman. Knocking-down of these fields exposes fields to leeward infection [Carter (22)]. Hand-weeding of *Emilia* in young fields will also expose nearby pineapple plants to infection, especially if the weeds are left in the field.

Weeds in many cultivated crops serve as reservoirs of both viruses and insect vectors, but the economic problem is to prevent the growth of these weeds altogether, since their control after establishment of vector populations again forces interhost movement and consequent virus spread. Chemical methods of weed control, especially the pre-emergence type, have greatly improved in recent years.

Host-plant sequence is in many cases governed by natural plant associations and their succession to climax associations. The factors of plant ecology must therefore be integrated with those of the insect's ecology, and the best documented case of its kind is that of the sugar-beet leafhopper and its weed hosts. As Cook (24) pointed out, the breeding grounds of the leafhopper are characteristically in arid country with a mean annual rainfall of 10 to 12 inches. All but one (along the Rio Grande in Texas and New Mexico) are west of the Continental Divide (Fig. 1). Since the early work of Carter (21) recognized the importance of weed-host succession in determining and maintaining populations of the leafhopper, there has been a continuous study made by many workers, whose extensive findings can be referred to only in summary [Douglass & Hallock (47); Fox (50, 51, 52); Lawson & Piemeisel (81); Piemeisel (89, 90, 91, 92); Piemeisal & Chamberlin (93); Piemeisel & Lawson (94); Piemeisel *et al.* (95, 96)]. When the original vegetation of the San Joaquin valley in California was mapped, there were five vegetation types recognized. All have been greatly modified by cultivation and overgrazing. Plant species replacing the original vegetation are determined by land use. Overgrazing results in a stand of winter annuals and, if too heavy, in bare soil. Intermittently farmed lands produce large areas of summer annuals. The beet leafhopper in this area utilizes both winter and summer annuals, and it is on these hosts that the huge populations of the insect develop.

Piemeisel and his co-workers have shown conclusively that, with proper protection, the original vegetation can be restored. Figure 2 is, in essence, a summary of their conclusions.

FIG. 1. Major breeding areas of the beet leafhopper and sugarbeet areas affected by them. Arrows indicate the general direction of spring movements of the insects. (Map courtesy of the Agricultural Research Service, United States Department of Agriculture.)

In southern Idaho, a seven-year study by Piemeisel (90) showed essentially the same type of plant succession but with special emphasis on fluctuations in dominance of the annual weeds. Lawson & Piemeisel (81) followed with a similar study conducted in the San Joaquin valley.

Changes in leafhopper populations following these fluctuations were followed in a parallel study by Fox (50), who found that, when the plant suc-

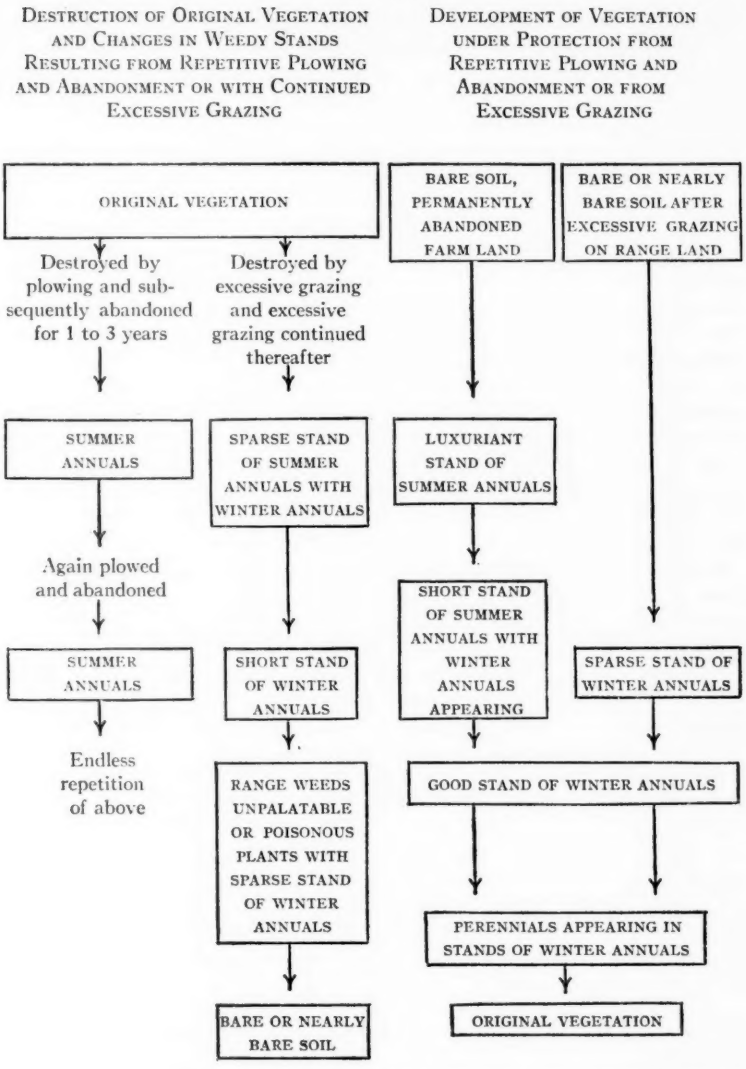
DESTRUCTION OF ORIGINAL VEGETATION AND CHANGES IN WEEDY STANDS RESULTING FROM REPETITIVE PLOWING AND ABANDONMENT OR WITH CONTINUED EXCESSIVE GRAZING
DEVELOPMENT OF VEGETATION UNDER PROTECTION FROM REPETITIVE PLOWING AND ABANDONMENT OR FROM EXCESSIVE GRAZING
ORIGINAL VEGETATION
BARE SOIL, PERMANENTLY ABANDONED FARM LAND
BARE OR NEARLY BARE SOIL AFTER EXCESSIVE GRAZING ON RANGE LAND
Destroyed by plowing and subsequently abandoned for 1 to 3 years
Destroyed by excessive grazing and excessive grazing continued thereafter
SUMMER ANNUALS
SPARSE STAND OF SUMMER ANNUALS WITH WINTER ANNUALS
LUXURIANT STAND OF SUMMER ANNUALS
Again plowed and abandoned
SHORT STAND OF SUMMER ANNUALS WITH WINTER ANNUALS APPEARING
SUMMER ANNUALS
SHORT STAND OF WINTER ANNUALS
SPARSE STAND OF WINTER ANNUALS
Endless repetition of above
RANGE WEEDS UNPALATABLE OR POISONOUS PLANTS WITH SPARSE STAND OF WINTER ANNUALS
GOOD STAND OF WINTER ANNUALS
PERENNIALS APPEARING IN STANDS OF WINTER ANNUALS
BARE OR NEARLY BARE SOIL
ORIGINAL VEGETATION

FIG 2. Diagrammatic representation of the changes in the stands that follow the destruction of the original vegetation. The left half of the figure deals with the changes as a result of continuous disturbance, and the right half with the changes taking place under protection from disturbances and leading to a re-establishment of the original cover [Piemeisel & Lawson (94)].

cession had progressed to the point where downy chess (*Bromus tectorum* Linnaeus) and sagebrush were dominant, the area lost its status as a leafhopper breeding ground.

Forty-three host-plant species were studied by Douglass & Hallock (47) to determine their relative importance. Host plants varied in their suitability for egg laying and there were differences in the nymphal populations of various species as the summer progressed.

Host-plant succession is not necessarily one of breeding hosts. Many are transient feeding hosts, although the insect may feed long enough to transmit viruses. Swenson & Nelson (128) reported no colonization by 18 species of aphids, yet mosaic of gladiolus was freely transmitted, and six new aphid vectors of cantaloupe mosaic virus were discovered in the process. Few, if any, of the 50 species of aphids transmitting yellow dwarf on onion actually breed on the plant. Dickson *et al.* (39) demonstrated that *M. persicae* did not colonize on the melon crop but is nevertheless responsible for the spread of cantaloupe mosaic in the desert valleys of California. Of the economic crops attacked by tomato spotted wilt virus, none are favorable hosts of the thrips vectors. The coincident geographic range of western yellow blight of tomatoes and curly-top of sugar beets was known for years before the co-identity of the two diseases was recognized [Shapovalov (114)]. The reason for this is that *C. tenellus* does not breed on tomato, although the feeding of transient leafhoppers can be economically devastating. Severin (102) reported that many hosts in which the leafhopper oviposited were unsuitable for development of the nymphs to the winged stage. Many other plants, recorded as hosts, served only as holdover hosts in times of stress, particularly during the short period in the fall between the drying up of the summer hosts and the germination of the winter annuals [Lawson & Piemeisel (81)].

The transfer of insects from endemic to introduced hosts, when the endemic hosts are virus-infected, can create a new host-vector-virus relationship that is extremely favorable to the insect and critical for the introduced economic crop plant. Native hosts of the swollen-shoot virus of cacao in West Africa are the endemic forest trees. Three families of the Tiliales, and the Malvacae have thus far been implicated. [Dale & Attafuah (30); Posnette *et al.* (97); Tinsley & Wharton (130)]. These forest trees stand as solitary giants in the forest; the under-growing cacao trees form an almost continuous canopy through which the mealybug vectors can move unhindered, except for barriers imposed by occasional breaks in the continuity of the canopy.

Study of the host-plant and environmental preferences can be used for vector separation. Hutchinson (70) worked with two leafhopper species, *Scaphytopius magdalensis* (Provancher) and *Scaphytopius verecundus* (Van Duzee). These insects had been reported to be covectors of blueberry stunt virus. Externally, the insects are similar and are separated morphologically by the male genetalia. Surveys, using sticky traps and sweep nets, showed that *S. magdalensis* was abundant in blueberry fields but absent from cran-

berry bogs. The converse was true of *S. verecundus*, which also prefers open, sunny environments to the dense growth of blueberry fields. The conclusion was reached that the *S. magdalensis* is the vector of blueberry stunt.

A somewhat similar situation, not yet completely clarified, is described by de Fluiter [(36); de Fluiter & van der Meer (36a)] in connection with *Rubus* stunt. The known vector is *Macropsis fuscula* Zetterstedt. Interhost transfer of the insect between raspberry and blackberry failed; therefore, to account for the failure of *M. fuscula* to infect cultivated blackberries, although it breeds on several species of wild blackberries, it was suggested that either two host-limited strains of the leafhopper, or a closely related species, exist. A related species, *Macropsis scotti* Edwards, is common on cultivated and some wild blackberries but cannot complete development on raspberries; its status as a vector is not yet reported.

Before *Jassus indicus* (Walker) was determined to be the vector of spike disease of sandal, Rao (98) recognized three area types coinciding with incidence of the disease: (*a*) the dry scrub jungle containing more or less dense stands of sandal and heavy undergrowth; (*b*) pole forests with only sparsely growing sandal and extremely variable undergrowth; and (*c*) cultivated lands, private estates, and village sites, where large and healthy isolated trees of sandal are frequent, with no undergrowth. The disease is most severe in the dry scrub, less so in the pole forests, where it spreads very slowly, and almost entirely absent in the cultivated lands, private holdings, and village sites.

### The Physical Factors of the Environment

The physical factors of the environment affect the vector populations in various ways. In the temperate and, to a lesser extent, in the subtropical zones, successful hibernation is the first requisite of a population build-up during the following active season. Man sometimes provides the suitable hibernation places. Smith & Brierley (120) report that bulb storage houses not only permit aphids to survive, but actually to transmit viruses to sprouting bulbs during that period. Mangold and fodder beets serve as hibernation quarters for aphids when the roots are overwintered in "clamps"—piles covered with straw and soil. Glasshouses are also a means whereby vectors are protected during the winter. Severe winters and cold spring weather are a major deterrent to many species. Carter (21) showed the correlation between severe winters and relative freedom from beet leafhopper outbreaks the following year. Later workers extended this to include the influence of spring weather. Annand *et al.* (2) reported the necessity of taking the spring weather into account, since this determines the time of the spring movement into cultivated fields. Fox (52) considered the time of the spring movement of more importance than the peak population.

Cook (25) used temperature summation to determine the spring movement of the beet leafhopper in the San Joaquin valley. Although Cook interpreted the correlation between degree days and spring flights solely on the

development of the insect and on the assumption that maturity was the principal stimulus, it is probable that the temperature summation related to plant development and maturity would coincide in most years.

When the hibernating insect is the vector, as is the case with the two species of *Piesma*, *P. quadrata* (Fieber), the vector of beet leaf curl, and *P. cinerea* Say, the vector of savoy, winter conditions in the unploughed areas, headlands, and woodlands where the insect hibernates will determine the extent of survival and the degree of infection in beets the following spring [Coons *et al.* (26); Wille (137)].

Low temperatures in spring were shown by Shands *et al.* (108) to markedly affect the survival of stem-mother aphids; this was not as a result of sudden drops in temperature below survival levels, but rather the general temperature levels during the developmental period. These same temperature conditions also reduce the amount of favorable foliage.

The severity of winter weather has a marked effect on the spring populations and migrations of *M. persicae*. During mild winters or in well-protected locations, the viviparous females can overwinter successfully on crops other than the true winter hosts. During severe winters, the overwintering females are eliminated, so that the population in the spring must develop from eggs laid on the winter hosts (peach and apricot, etc.). Populations of aphid species that overwinter only in the egg stage are not as susceptible to variations in the severity of winters [Watson *et al.* (134)].

Hull & Watson (69) reported that sugar-beet yellows develops seriously only after a succession of mild winters. The first mild winter confines infestations largely to seed crop areas, but, if the following winter is also mild, then some plants of the heavily infested root crop remain in the ground, together with infected mangel, spinach, and spinach-beet. These serve as widespread sources of infection for the next succeeding year.

While climate will determine the geographic range of an insect, weather is responsible for the fluctuations that determine its seasonal activity and numbers. Even in tropical West Africa, where weather seems to be extremely uniform, seasonal differences in temperature associated with rainfall conditions materially affect mealybug vector populations. The decline in populations during the first part of the year is associated with periods of increasing rainfall. Maximum populations occur in October and November, but changes during the year are of a five- or sixfold order, and the activities of parasites and predators, again related to season, are held to be important [Cornwell (28)].

*Cataclysmic weather conditions.*—Cataclysmic weather conditions sometimes affect vector populations. Shands *et al.* (106) reported that the two hurricanes in northeastern Maine so reduced aphid populations that feeding damage was not economic; fall populations of *M. persicae* were so reduced on both potato and winter hosts, that reduction of populations occurred the following spring. The hurricanes occurred when the fall migrants were beginning to mature and disperse to primary hosts. The effect on the buckthorn

aphid was not so pronounced because, on the primary host (*Rhamnus alnifolia* L'Hértier), populations were already large, egg deposition had started, and the plant is low-growing and in sheltered environments.

Staples & Allington (122) reported that hail, occurring just before wheat harvest, forced a volunteer crop of wheat. A wheat mosaic epidemic followed because of heavy infestations of *Aceria tulipae* (Keifer).

*The use of phenology.*—Phenology, that is, the study of recurring natural phenomena in relation to climatic conditions, deserves more attention than it gets. Its great advantage is that the phenomena it measures are summaries of many individual factors that are difficult, if not impossible, to interrelate in any other way. The disadvantage is that, although the value of phenological data improves as the years of observation are increased, very few individuals are in a position to maintain these data long enough.

Virus disease incidence is the phenological climax of phenomena of virus sources and their availability to the insect, the susceptibility of the virus host plant, and vector-insect numbers (including the insect's movements into and distribution within cultivated crops, its vector efficiency, and its relationships with the viruses it carries). It is reasonably certain that for no plant virus disease are all the contributing factors and their interrelationships fully understood.

The strawberry aphid [*Pentatrichopus fragaefolii* (Cockerell)] has two rhythms in its annual cycle, depending on climatic conditions during the four seasons. De Fluiter (36) found that, with a combination of severe winter, bad spring, and early summer weather, populations of the aphid reach only a low peak by autumn, and these are soon overtaken by frost. Under the converse conditions, i.e., mild winter, favorable spring, and early summer weather, the first peak of population occurs in late June and early July. This then declines rapidly, but rises again in late September and October. Periods of alate production occur in May and June and October to early December, and the population increases coincide with the production of young strawberry leaves.

## The Virus Source

The virus source may be the hibernating insect itself. Curly-top is known to overwinter in its insect host, *C. tenellus* [Wallace & Murphy (133)], although the availability of winter hosts of the insect might affect this. If the hosts are immune to the virus, then the leafhopper may lose its infective "charge." Severin (103) suggested that infective power was not retained by adult female leafhoppers unless they could derive new sources during the winter. When the spring annuals germinate in the fall, there is ample opportunity for the leafhopper to feed intermittently on infected plants. The two beet viruses transmitted by species of *Piesma* presumably also overwinter in the insects.

The most important sources of virus, however, are infected plants, and Severin (103) listed 14 species of annual plants and three perennials in which

the virus of curly-top overwintered in California, and 75 annuals and four perennials that were susceptible (103). Wallace & Murphy (133) studied the epidemiology of the disease in southern Idaho, where 2,000,000 acres of primary wild host area is supplemented by 9,000,000 acres of secondary overgrazed sagebrush area. Infection of the spring brood of leafhoppers and the maintenance of virus reservoirs throughout the summer and fall are assured by the susceptibility of the principal hosts, mustards and Russian thistle. Beets left in the field after harvest survive the winter and are important sources of virus if the field is replanted to beets the following year. In the Imperial Valley in California, with its extremely high summer temperature, Flock & Deal (49) found that sugar beets and weeds in the cultivated areas were the most important sources. Aster yellows and Pierce's disease of grapes, both leafhopper-transmitted, also have a long list of wild hosts which serve as reservoirs [Freitag (53); Kunkel (79)]. Anderson (1) sampled 150 species of plants as possible field sources of five pepper viruses; 79 of these were positive for one or more virus, and five viruses were recovered from about 21 plant species. Chokeberries are a reservoir of X disease of peach [Hildebrand & Palmiter (62)]. Seed crops of biennials are important sources, i.e., sugar beet yellows and beet mosaic. With some viruses, however, the most important sources are diseased plants within the crop itself [Broadbent (15); Broadbent *et al.* (18); Doncaster & Gregory (42)].

*Susceptibility to virus infection.*—Susceptibility frequently decreases as the plant ages. Wallace & Murphy (133) considered that the age of sugar beets at the time of infection was the dominant factor in the epidemiology of the disease. Hills & Taylor (66) showed that cucurbits infected with curly-top in the cotyledon, or two-leaf, stage were killed, but infection later than this resulted in stunting or growth-retardation only. Shands *et al.* (107) recognized the need for protecting potato plants in the early stage of leaf emergence, that being the critical time for establishment of early aphid migrants. Unfortunately, the phenology of the crop is also close to that of the vector, and cultural modifications have their definite limitations. Broadbent & Doncaster (17) recognized that migration time coincided with development of the host plants. Sometimes, however, a very small time difference is important, as Hansen (56) found with sugar beet yellows. April 1st, 15th, and May 1st plantings were 38, 53, and 70 per cent infected, respectively. Whether it was practical for planting to be finished by April 1st and whether the farmer knew any good reason why it should have been are quite different matters.

Sometimes advantage is taken of the early attractiveness of crop plants. Border rows of sugar beets are planted ahead of the normal planting season in the areas likely to be affected by *P. quadrata*. These serve as a trap for the insects coming in from their hibernation quarters, and they can be destroyed, either by crushing the young infested beets or, more recently, by the use of insecticides before the normal planting is made.

There are many other factors affecting susceptibility, but they are physio-

logical rather than ecological. It is of interest, for example, to know that modifying day length will affect susceptibility, but the sun appears to be rather regular in its habits.

### The Size of Vector Populations and Their Movements

The size of vector populations and their movements are of critical concern in disease incidence. Davies (34) used an index figure in the evaluation of areas suitable for potato seed growing. Where there was a rapid increase of virus disease among potato stocks, index counts of *M. persicae* per 100 leaves always exceeded 100; where there had been no increase of virus infection, the figure did not exceed 25. For such an index to be valid, the index should reflect the magnitude of initial infestations of alates from outside sources and their subsequent spread, and not merely an index of the rate of reproduction of aphids within a field.

Henner & Schreiner (61) found some correlation between total numbers of *M. persicae* on potato leaves and the incidence of virus disease, but some low counts were obtained (less than 20 per leaf) in certain areas of high disease incidence. Broadbent (12) found a correlation between potato leaf-roll incidence and maximum counts per 100 leaves of *M. persicae* in England and Wales. Spread, however, was better correlated with the number of alate *M. persicae* caught on sticky traps than with total number from leaf counts.

Watson & Healy (135) derived a mathematical formula to account for the observed incidence of beet yellows virus in sugar beets in England. Assuming that the crop is visited by $N$ aphids at a time when the proportion of infected plants is $k_0$, the predicted proportion of infection for a time three or four weeks later is: $k_1 = k_0 + 100(1-k_0)(1-e^{-NI})$, where $I = p[(1-k_0)^t + k_0 t - 1]/k_0$. This formula adequately accounts for the observed spread of infection when $N = 1/10$ the sticky trap count for three to four weeks preceding the time $k_0$ infection is observed; $p$, the probability of infection by a single aphid equals .5; and $t$, the number of movements per aphid effective for spreading beet yellows virus equals 5. *M. persicae* populations were not, however, correlated with the incidence of beet mosaic virus. Similar differences have been shown by Doncaster & Gregory (42) and Broadbent (12) with *M. persicae* populations in the correlations of incidence of potato leaf-roll and potato virus Y. Leaf-roll can be correlated, but not virus Y. While virus-vector relationships are not in review here, it can be mentioned in passing that the explanation for the differences in correlation are to be found in the fact that beet mosaic virus and potato Y virus are both non-persistent in the vector and therefore quickly dissipated. Dickson *et al.* (40) measured populations of flying aphids in southern California citrus groves and related these to the spread of tristeza (quick decline) virus. The rate of spread of the virus was about two new infections each year for each diseased tree. The green citrus aphid (*A. spiraecola*) constituted 85 per cent of the aphid catch; the melon aphid (*A. gossypii*), 3 per cent of the catch. Although *A. spiraecola* is reported to be a vector of the virus in Florida, it is not known as such in

California. There, the melon aphid is the vector, although its efficiency is much below that reported for *A. spiraecola*. However, with 35,000 individuals flying to or from a single citrus tree in a single year, natural spread of the virus can be fully accounted for, even though an average of 5600 melon aphids is required to produce each experimental infection.

## Estimates of Vector Populations

Estimates of vector populations, coupled with phenological observations, have become considerably important in the development of advisory services to growers. Shands and his associates (109) have, for many years, followed the development of *M. persicae* populations on wild plum thickets and their migrations as well as development on potatoes. This information has been relayed to the farmers through the county agricultural agents. Timing and spray frequency have in this way been co-ordinated and adjusted to seasonal differences.

Wet and stormy springs may so reduce aphids on sugar beets that spraying may not be necessary, but this is determined by the arrival of winged aphids in the beet fields. In areas where beet yellows is prevalent, one aphid to four plants calls for a spray warning; in other areas, one aphid to one plant is the standard, but no warning may be necessary if this level is not reached by mid-July [Hull (67, 68)].

Hille Ris Lambers (63) bases an advisory service to potato seed growers on the knowledge that 97 per cent of the migration of *M. persicae* from winter hosts to summer hosts comes from peach and *Prunus serotina*. Estimates of population size are based on the number of fundatrices found annually, together with counts of predators. The exact time of migration is known, so that the degree of early spread of leaf-roll can, to some extent, be predicted by multiplying the number of primary colonies by the number of alatae per colony. Confirmation is obtained by direct counts of alatae arriving on potato during the migration period. This applies to early spread caused by the alatae flying from the winter hosts. The main spread, occurring in July, is by alatae developed on potatoes and other herbaceous plants. Like Broadbent (12), Hille Ris Lambers found no correlation between the number of aphids on the plants at that time and virus spread in the fields where the counts were made. The correlation is with trap catches and the data from a large number of these are used to prescribe dates for early lifting of the seed potato crop. Since a few days time can involve serious losses, trap catches are dealt with the day of arrival. Munster (87) has described methods of determining aphid development with a similar objective in view.

Broadbent (12) found a correlation between numbers of alate aphids caught on sticky traps and the spread of leaf-roll. Rugose mosaic spread was not so clearly correlated, because a number of vectors were involved, but the average health of potato stocks for the following year was predictable on the basis of the average trap catch data. Hauschild (57) has derived a theo-

retical formula, which is based on the initial health of the crop and the course of the aphid infestation, for use in a similar prediction.

In the case of curly-top of beets and other susceptible economic crops, the importance of the spring movement of leafhoppers into the cultivated fields depends on the data and duration of the movement and on the size of the incoming population. The size of the population depends on the size of the population dispersing from Russian thistle in the fall to winter and spring hosts, the winter mortality, and the condition of the surviving females. The number of spring generation leafhoppers entering cultivated fields depends on the location of the cultivated area *vis-à-vis* the breeding grounds, dispersal as determined by wind movement, and time of dispersal as determined by temperature and host-plant abundance.

Fisken (48), first determining that the principal overwintering area for *M. persicae* was in the market garden area around Edinburgh, showed by trap counts that the initial infestation in crops within different areas did not differ materially between seasons, but that a hot, dry summer brought earlier infestation and earlier maximum populations. Distance from the dispersal area and the lateness of season are perhaps more important than actual numbers in determining relative freedom from leaf-roll and Y viruses [Fisken (48)].

Fox (52) posed the question as to what constitutes an economic population of beet leafhoppers. This is determined by several factors: the weather during the spring, the percentage of leafhoppers carrying virus [Wallace & Murphy (133) report variations from 4 to 67 per cent from one year to another], and the age and size of the sugar beets at the time. With very early movements, severe damage occurs; later movements cause little or negligible damage, and the time of the spring movement is more important than the population peaks. In the writer's experience, however, the most damaging years have been those where early spring movement and large populations coincided.

When the invaded host plant is not a favorable host for the vector, disease incidence depends entirely on the size of the invading population. Percentage disease incidence will, therefore, depend on plant density and, within the limits imposed by good agronomy, the percentage of diseased plants can be reduced by increasing the density of planting. Van der Plank & Anderssen (131) showed this connection with tomato spotted wilt on tobacco. Increasing the number per acre $n$ times reduced the proportion of diseased plants from $1-q$ to $1-\sqrt[n]{q}$ where $q$ is the proportion of healthy plants at standard density. Linford (83) reported a similar conclusion with the same virus in pineapple, thus confirming Carter's conclusion (22) that even infield populations on favorable weed hosts were not a factor in the disease incidence in pineapple plants in that field. Curly-top in tomato is a similar case of density of planting determining the percentage of plants infected [Shapovalov *et al.* (115)].

Peasant cultivators in East Africa used poor agronomy in their growing of groundnuts (peanuts). Spacing was much too close, and weeding was delayed, but the method had the pragmatic sanction. When more open spacing and clean culture were tried, groundnut rosette increased to serious proportions [Storey (123)].

There are many other examples of the effect of plant spacing on disease incidence; Broadbent (15), Blencowe & Tinsley (8), and Shirahama (117) all reported such effects with aphid-transmitted viruses, even where the crop plant was a suitable vector host.

## Physiological and Behavior Factors

Physiological and behavior factors are the handmaidens of autoecology, so are appropriately mentioned here. Cauliflower mosaic virus is in high concentration in new leaves produced by infected plants, while cabbage ring spot virus occurs mainly in older leaves and, even there, is localized to the symptom areas. Only in recently infected plants does cabbage ring spot virus occur in young leaves. The two aphis vectors, *M. persicae* and *B. brassicae*, alight and feed on the upper portions of the plant and are less likely therefore to acquire cabbage ring spot virus and cauliflower mosaic virus. The older leaves of cabbage are in a more favorable position to induce the alighting of aphids than those of cauliflower, which may explain the more frequent infection of cabbage with the ring spot virus [Broadbent (14)]. There are differences in the reports on the influence of growth on aphid development. Bald *et al.* (5) reported that aphids avoid infected plants because of stunting caused by infection. Hull & Watson (69) reported that vigorous, well fertilized plants were more severely infested. On the other hand, according to Arenz (4), *M. persicae* grew faster on potatoes with leaf-roll and severe mosaic. *Aphis fabae* Scopoli reproduces poorly on active presenescent leaves [Ibbotsen & Kennedy (71); Kennedy (75); Kennedy *et al.* (76)]. *C. tenellus* develops larger populations on diseased plants than on vigorous, densely foliaged, healthy ones [Carter (21); Romney (99)]. *T. tabaci* populations on diseased *Emilia* are higher than on healthy *Emilia* [Carter (22)], and Severin (104) reported that leafhoppers developed faster, lived longer, and reproduced more on the diseased than on the healthy plants. There is one case reported [Jensen (71a)] where a plant virus is fatal to its vector, but that is a virus-vector, not a host plant-vector, relationship. No explanation has been offered for the beneficial effects of the diseased plant on the insect, but presumably it can be ascribed to a higher concentration of cell sap and, probably, a higher proportion of carbohydrates, providing a more efficient diet for growth and reproduction.

*Flight behavior.*—This is a somewhat controversial subject. For many years, the "flight activity" hypothesis was generally accepted. Essentially, this hypothesis ascribes the governing factors of aphid flight to the physical factors of wind, temperature, and humidity. Johnson (73), however, in a detailed analysis of this hypothesis and a reconsideration of the supporting

data, has presented another hypothesis concerning flight from breeding sites which is based on physiological factors, behavior being secondary. Moulting of the winged nymphs into alatae, usually most intense early in the morning, is followed by one or more additional peaks during the day. Flight maturity of these newly emerged alatae is usually reached in 24 hours, after which they fly away in "flushes" on the first migratory flight. Since aphids lose their ability to fly, owing to autolysis of the flight muscles after a few days [Johnson (72)], their ability to spread virus diseases subsequent to the first migration is considerably impaired. Changes in the number of aphids in the air throughout the day and from day to day are, therefore, actually collective-population changes.

Dickson (38) ascribed the high efficiency of *M. persicae* in the spread of cantaloupe mosaic to the flight behavior of the aphid. Although the insect does not colonize the plant, it swarms over the crop in short flights, with short periods of feeding. Swensen & Nelson (128) consider that whether the number of different species that alight on a crop plant are proportional to the number of those species that fly over the crop is still a moot point.

Finally, some reference should be made to the complexity of some biocoenoses involved in virus transmission. Perhaps the most elaborate of these is that concerned with swollen shoot of cacao. According to Strickland (125, 126), no less than 17 species of pseudococcids, 75 species of ants, and three arachnoids are involved. There are three distinct ecological niches: (*a*) the association between mealybugs and myrmicine ants; (*b*) mealybugs of the genera *Paraputo* and *Formicoccus* and the wild host trees; and (*c*) a negative association between myrmicines, and *Oecophylla* and *Macromischoides*.

*Pseudococcus njalensis* Laing is always attended by ants and *Crematogaster* density is closely correlated with mealybug density. Tree to tree variation in mealybug density is largely dependent on the identity of the dominant ant species. There is no direct correlation between mealybug density and incidence of swollen shoot. Mealybug populations in the devastated areas are very low compared with areas of active spread.

In conclusion, it can be said that these ecological aspects are an essential part of the biological approach to plant viruses.

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# MECHANISM OF TRANSMISSION OF VIRUSES BY MOSQUITOES[1]

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## Introduction

Previous reviews by Day & associates (34, 35, 36) have treated extensively the mechanisms of transmission of plant and animal viruses by various arthropods in many groups. It is not the purpose of the present paper to be repetitious in again attempting such broad coverage but rather to consider in some detail recent findings and hypotheses concerning the mechanism involved in the transmission of viruses by mosquitoes alone.

The vector-pathogen relationships categorized by Huff (51) and discussed by Day (34) in respect to arthropod-borne viruses are still acceptable and convenient. These are *mechanical transmission*, in which the vector is merely a carrier, usually by contaminated mouthparts, from infection source to host, and *biological transmission*, in which the arthropod plays a necessary biological role in the maintenance of the pathogen. Virus transmission by both of these methods can be accomplished by mosquitoes and will be discussed. However, since the biological method is of far greater epidemiological significance, as well as more complicated, it will be considered in greater detail. The present paper will be concerned primarily with analysis of the step-by-step development and progression of virus within mosquitoes "biologically" infected.

## Mechanical Transmission

Various types of mechanical transmission have been described by Day & Fenner (37) to account for peculiarities of transmission encountered in certain plant viruses, but, in the mosquito-borne virus group, mechanical transmission appears to be of the simplest or "flying pin" type. Work by Fenner, Day and associates (38, 39) attests the importance of this type of transmitting mechanism in the spread of myxoma virus in rabbits by mosquitoes. They have demonstrated that the transmission of this virus by mosquitoes is strictly mechanical and that the mosquitoes are incapable of becoming biologically infected.

The expressed thesis of Day's review (34) was that:

> all virus-vector relationships so far described are covered by these [mechanical and biological transmission] categories, that no other of the possible mechanisms have been involved, and that no virus has been shown to be transmitted by more than one mechanism.

[1] The survey of the literature pertaining to this review was concluded April 1, 1960.

The statement implies that biologically transmitted viruses are never transmitted mechanically in nature. However, it is theoretically possible for any blood-feeding arthropod to be a mechanical vector under favorable circumstances; namely, a high concentration of virus in peripheral areas of the vertebrate host and close association of the arthropod with the host. The critical virus level required would vary according to the susceptibility of the next host, the amount of virus carried by the vector, the stability of the virus, the degree of protection furnished the virus by the vector to prevent its inactivation, the time period between feedings, and the temperature and humidity at the time of virus transfer. Even such factors as mouth-cleaning habits and probing activity of individual vector species could alter the efficiency of mechanical transmission (22). The frequency of occurrence of infectious levels of virus in blood or excretions of infected animals together with the population density of mechanical vector species and of closely associated normal susceptible vertebrate hosts are limiting factors in extending mechanical transmission beyond a mere occasional occurrence of no particular epidemiological significance.

Recent studies indicate that, under some circumstances, mosquitoes may serve both as biological and mechanical vectors of the same virus. This was first observed in an investigation of the effects of temperature upon the extrinsic incubation period of eastern encephalitis virus in *Aedes triseriatus* (Say) (22). Groups of mosquitoes were infected with eastern encephalitis virus by feeding upon viremic chicks, then were incubated at either 70°, 80°, or 90°F. Series of mosquitoes were removed from each incubation group at three, six, nine, 13, and more days of incubation, and their ability to transmit was determined by feeding them individually upon normal chicks. When incubation was at 70°F., 60 per cent transmitted on the third day of incubation, and 14 per cent on the sixth day. In view of the short periods of incubation and the decreasing efficiency with time, these transmissions were considered to be mechanical. On the ninth day, only 4 per cent transmitted, which was interpreted as the last of the mechanical transmission or the beginning of biological transmission. Thereafter, the transmitting efficiency progressively increased, to reach a peak of 80 per cent on the thirty-fourth day of incubation. Evidence of early mechanical transmission was not as clear when the incubation was at 80°F., probably because of a more rapid die-off of mechanically carried virus, a more rapid development of biologically transmitted virus, and a more thorough beak-cleaning by the more active mosquitoes. Even so, the transmission rate at three days was higher than at six days, 21 per cent versus 14 per cent. Subsequent rates raised rapidly to over 80 per cent by 17 days. No evidence of mechanical transmission was seen in the 90°F. group, presumably because of fast virus die-off at that temperature and masking by rapid biological development.

Another study (21) proves the ability of *A. triseriatus* to transmit eastern encephalitis virus mechanically. Transmissions by interrupted feedings were compared with transmissions by jabs of infected pins. A considerable number

of insect pins were infected by running them one-fourth inch under the skin of a viremic chick. The mosquitoes were similarly "infected" by permitting them to probe briefly upon an infected chick (without taking visible blood). Pins and mosquitoes were then incubated at 80°F. and 75 per cent relative humidity. Individual pins and mosquitoes were rejabbed or induced to reprobe into normal chicks, either immediately or after one, four, 20, or 70 hours. The results (Table I) revealed no significant difference between the pins and the mosquitoes as mechanical transmitters. Transmissions were accomplished by both methods for up to 70 hours. Similarly, *Stomoxys calcitrans* (Linneaus) (the stable fly) transmitted eastern encephalitis virus up to four hours.

TABLE I

COMPARISON OF PINS AND *AEDES TRISERIATUS* AS MECHANICAL TRANSMITTERS OF EASTERN ENCEPHALITIS VIRUS

| Interval between infecting and transmitting probe* | Transmissions/Attempts | |
|---|---|---|
| | Pins | Mosquitoes |
| 0 | 5/5 | 10/10 |
| 1 | 5/5 | 9/10 |
| 4 | 10/10 | 7/10 |
| 20 | 4/10 | 12/20 |
| 70 | 1/20 | 2/20 |

* Shown in hours.

Barnett has shown that western encephalitis virus can also be transmitted mechanically. In his studies, *Culex tarsalis* Coquillett transmitted the virus from an infected to a normal chicken by means of interrupted feedings (2).

Evaluation of the possible epidemiological importance of mosquitoes and biting flies as mechanical vectors of normally biologically transmitted viruses is difficult at best. It would appear, however, that transmissions of Venezuelan equine encephalomyelitis virus from horse to horse could easily be caused by interrupted feedings, since this virus is highly infectious for horses, occurs in their blood in exceptionally high titer, and is relatively resistant to inactivation (61, 77). Even eastern and western encephalitis viruses, although usually producing only low viremias in horses, might occasionally be transmitted in this manner (60, 78). One cannot review the great epizootics of western encephalitis virus in horses throughout western United States (12, 76) without feeling that mechanical transmission may have contributed to the rapid virus spread. A similarly explosive outbreak of eastern encephalitis virus occurred in southwestern Louisiana in 1947 and resulted in the death of several thousand horses (50). High populations of avid mosquitoes, such as *Aedes* and *Psorophora* species, and of pestiferous deerflies (*Chrysops*) and

horseflies (*Tabanus*), common in the Louisiana area, plausibly could have played a part as mechanical transmitters.

That biting midges may also serve as mechanical vectors is suggested by the isolation of eastern encephalitis virus from *Culicoides* species in Georgia (58) and of Japanese B encephalitis virus from *Lasiohelea taiwana* (Shiraki) in Fukien (95). J. R. Anderson and associates, University of Wisconsin (57), have made observations suggesting that *Simulium* species may have been involved in an outbreak of eastern encephalitis virus in young turkeys in Wisconsin.

In view of the high viremias of eastern (62) and western encephalitis (48, 63) known to occur in birds, these viruses are probably, on occasion, spread mechanically by mosquitoes from a single bird to others in a rookery or nest. Possibly even yellow fever virus could be transmitted in this manner from an infected monkey to associated normal ones, particularly at their sleeping sites. In any event, however, mechanical transmission in the situations discussed above would be dependent upon active infection in the area produced by biological vectors and would thus serve only as a supplementary means of virus transfer.

## Biological Transmission

Biological transmission, holding more secrets than the mechanical type, has intrigued numerous investigators over the years and stimulated much research. As a result, contributions toward our knowledge of the behavior of viruses in mosquitoes have been considerable, despite the difficulties encountered. It is now possible to follow with some degree of certainty, although with frequent reliance upon hypothesis, the course of a virus from the moment of ingestion by the mosquito to its injection into a subsequent vertebrate host.

*Establishing the infection.*—The female mosquito draws up its food (blood, nectar, plant juices) by means of a muscular pharyngeal pump, and, apparently, stimulation of special sensory organs in the buccal cavity (33) determines the disposition of the meal in the digestive tract. The presence of intact blood cells is believed to stimulate a partial or complete contraction of sphincter muscles of the diverticula, causing the bulk of the meal to go to the hind portion of the mid-gut. The reverse occurs in non-blood meals, with the diverticula receiving all or most of the food upon closure of the sphincter of the proventriculus. Considerable variation in the degree of diversion into these different areas is apparent in different mosquito species and between individuals of the same species (87).

To the best of our knowledge, mosquitoes become infected in nature only through the ingestion of infected blood; the disposition of ingested carbohydrates thus is of little concern in the understanding of naturally acquired infection. In laboratory studies, however, the susceptibility of mosquitoes to various viruses has on occasion been determined in an artificial manner by the feeding of virus in sugar solutions. In the interpretation of results thus

obtained, the possibility should be kept in mind that diverticular tissue may be of a different susceptibility than tissue of the mid-gut. Such a difference has been suggested by the finding that up to 100 times as much eastern encephalitis virus in sugar solution as in blood was required to infect *Aedes aegypti* (Linnaeus) and *A. triseriatus* (20). Also, Hale *et al.* (43) have reported failure to infect *Culex tritaeniorhynchus* Giles with Japanese B encephalitis virus in sugared solutions containing no blood when only a low virus concentration in solutions containing blood was adequate.

Since the hind part of the mid-gut receives most or all of the ingested infected blood, it is logical to assume that initial infection occurs in cells of that area. That this is actually the case is supported by the finding of LaMotte (65) that Japanese B encephalitis virus occurred in high titer in the rear part of the mid-gut of *Culex pipiens* Linnaeus before it could be detected in tissues farther forward.

One of the main factors determining infection of the mosquito and its importance as a disease vector is the susceptibility of this mid-gut area to infection. Studies on yellow fever showed that ingestion of definite concentrations of virus were required to infect *A. aegypti* and *Haemagogus* species (1, 3, 4, 88). If these mosquitoes were fed upon virus in lesser concentration, they failed to become infected; if the concentration was increased, a higher proportion of infected specimens was obtained. Others [Barnett (2); Chamberlain and co-workers (17, 20, 23, 26); Gresser *et al.* (42); Hale *et al.* (43); Hurlbut (55); LaMotte (65); Thomas (85)], working with a number of viruses, including those of eastern encephalitis, western encephalitis, Venezuelan equine encephalomyelitis, St. Louis encephalitis, Japanese B encephalitis, and West Nile encephalitis, demonstrated that the "infection threshold," or virus concentration required to establish an infection in at least a proportion of the individuals ingesting it, varies considerably between mosquito species for the different viruses. For example, a relatively low concentration of western encephalitis virus can establish an infection in *C. tarsalis* (23), whereas 10,000,000 times as much rarely is adequate to infect *Culex quinquefasciatus* Say (20). With eastern encephalitis virus, a titer around $10^{3.0}$ per .03 ml. of blood is adequate to infect *A. aegypti*, but a titer of about $10^{8.0}$ is required for *Culex salinarius* Coquillett (20). Furthermore, a mosquito highly susceptible to one virus may be relatively insusceptible to another; for example, while *C. quinquefasciatus* is extremely susceptible to St. Louis or Japanese B encephalitis virus (26, 65), it is quite refractory to infection with eastern or western encephalitis virus. *Aedes* mosquitoes, in general, are infected with eastern or western encephalitis or Venezuelan encephalomyelitis with relative ease (17, 20) but with St. Louis encephalitis virus with some difficulty (26).

The threshold phenomenon is clearly a reflection of what has been termed by various workers a "gut barrier" to infection. Once this gut barrier, no matter how strong, is overcome by ingestion of virus in adequately high concentration, infection may result. For example, none of a group of *Orthopodo-*

*myia signifera* (Coquillett) became infected from feeding upon eastern encephalitis virus in a titer of $10^{6.6}$, but a high proportion were infected when the titer was $10^{7.7}$ (20). Other workers have had similar experience with Rift Valley Fever virus in *A. aegypti* (41). None of the mosquitoes became infected when fed on virus in a titer of $10^{7.0}$, but a high proportion did when the titer was $10^{8.0}$. It is apparent, therefore, that a high threshold does not necessarily portend a low-level infection once the infection is established.

If the gut barrier is overcome or by-passed, infection of various tissues in the rest of the mosquito body is readily accomplished. For example, direct intrathoracic inoculation of various species of mosquitoes has revealed a general susceptibility to all the biologically transmitted mosquito-borne viruses thus far tested (53, 54, 55, 67, 93). In fact, Hurlbut (56) has shown that even many non-hemophagous insects are susceptible by this direct inoculation method.

A number of hypotheses attempt to explain the mechanism of the gut barrier: virus inactivation by digestive fluids, impermeability of the peritropic membrane, variations in permeability of gut cell membranes, limited number of specific virus receptor sites on the gut cell, and a surface-type defense mechanism. Arguments for and against each can be presented, but none has been proven. Story's finding with a plant virus (corn streak)—that the gut barrier of *Cicadulina mbila* (Naudé) is controlled by a single, dominant sex-linked Mendelian character (80, 81, 82)—suggests that the barrier mechanism may be genetically determined.

Probably digestive juices are not entirely responsible, since inactivation of ingested virus is a relatively slow process. For example, no appreciable drop in titer of eastern encephalitis virus in the blood meal is demonstrable within the first half-hour after ingestion; even a day later at 80°F. a considerable amount usually remains (21). Furthermore, grinding a single infected mosquito in a pool with up to 100 normal ones does not appear to reduce the virus titer, although reduction might be expected if digestive enzymes had an immediate deleterious effect (21). Certainly digestive juices could not always account for a poor infection rate; in at least one instance, gut cells have been shown to be insusceptible even to virus introduced directly into the body cavity. When McLean (67) inoculated Murray Valley encephalitis virus intrathoracicly into *Anopheles annulipes* Walker, the virus content of the gut never increased beyond barest detectable amounts, although a high concentration was recovered from the rest of the body.

On the other hand, LaMotte (64) has shown clearly by microscopic sectioning that digestion commences on the periphery of the blood mass and that intact blood cells remain inside the mass for a considerable time. Rapid inactivation of surface virus by digestive fluids could go undetected by our methods of virus measurement because the amount inactivated might represent such a small proportion of the total virus present. It is surface virus that would be expected to infect the gut cells, not those virus particles trapped in the solidifying blood meal mass.

Waterhouse has demonstrated the presence of a peritrophic membrane in adult female *A. aegypti* and *C. quinquefasciatus* (90), and it probably occurs in other mosquito species as well. This membrane appears to be produced from a precursor material secreted by the lining cells themselves rather than from specialized cells at the fore-end of the mid-gut. Impermeability of the peritrophic membrane might prevent the virus from reaching the susceptible cells of the gut, since Schildmacher (74) has shown that colloidal gold particles of 2 to 4 mμ diameter diffuse through the peritrophic membrane of mosquito larvae, but particles measuring 20 mμ do not. The arthropod-borne viruses are generally believed to range from 15 to 50 mμ (79) and might thus be retained. However, this mechanism does not account for the high barrier of certain mosquito species against infection with one virus but a low barrier against another. With particle size of both viruses equivalent, equal rates of retention or diffusion would be expected. A similar argument can be presented against small pore size in cell membranes. Equal-sized viruses should have equal opportunities for passing through occasional "weak spots" or larger pores.

A more tenable explanation for a high threshold or gut barrier may be a limited number of specific virus receptor sites on the lining cells of the gut, thus requiring a high concentration of virus to ensure proper alignment and contact. A single mosquito species might have, for example, few eastern or western encephalitis receptor sites and require high concentrations of these viruses to establish infection but an abundance of St. Louis or Japanese B encephalitis receptor sites, permitting ready infection by low concentrations. One could test this hypothesis by attempting blockage by killed virus. On the other hand, the gut barrier might be an inhibiting substance on the lining of the gut which can be neutralized or "saturated out" by high concentrations of virus, permitting the remainder to establish infection. This hypothesis should also lend itself to testing by the feeding of mixtures of killed and live virus, but with an opposite result expected from that described above in the case of blockage of receptor sites with killed virus. Known inhibiting substances in vectors of plant viruses, leading to formation of enzyme-virus complexes, are noted by Day & Bennetts (36). They state, however, that, thus far, inhibiting substances in insects for animal viruses are unknown.

The infection threshold concept assumes considerable epidemiological importance when it is realized that virus levels attained in the blood of different vertebrate host animals vary considerably, species by species. The most favorable vector-host affiliations in nature would be those of a highly susceptible (low-threshold) vector and a high viremia host (73). There might be instances, however, when a relatively high threshold vector might be expected to assume considerable importance if it occurs in large numbers or is closely associated with hosts that produce adequately high concentrations of virus.

A correlation apparently exists between the thresholds of primary vectors and the viremias in important vertebrate hosts. If the viremias attained in

the hosts are relatively low, as with St. Louis or Japanese B encephalitis virus in birds (10, 18, 45, 47, 49), the threshold of the vector is also low; e.g., *C. pipiens* or *C. tarsalis* in St. Louis encephalitis virus [Chamberlain *et al.* (26)] or *C. tritaeniorhynchus* in Japanese B encephalitis virus [Gresser *et al.* (42); Hale *et al.* (43)]. If the viremias attained are high, as with Venezuelan equine encephalomyelitis virus in horses (61) and eastern encephalitis virus in small birds (62), the threshold is also relatively high (17, 20). This correlation appears to be a reflection of a natural selection process. Perhaps there is a selection for virus strains producing an adequate viremia; low viremia strains could not survive as they could not infect more mosquitoes and thus would not be retransmitted. Selection in the upward direction would tend to level off once an adequate infecting level for the primary vector was attained [Chamberlain (14); Reeves (71)].

*Virus multiplication.*—Davis *et al.* (31), upon grinding and titrating *A. aegypti* various days after infection with yellow fever virus, found an initial decrease over that ingested, followed by an increase, but never to a level surpassing that originally ingested. This they interpreted to indicate a latent or adsorbed stage followed by virus release, but not actual virus multiplication. Their findings were reinterpreted by Sellards (75) to indicate initial virus decrease, infection of the insect, and subsequent multiplication. Further studies with a number of viruses, e.g., yellow fever (91) and eastern (15, 22), western (85), St. Louis (26), Murray Valley (66, 67), West Nile (28, 29, 55), and Japanese B encephalitis (43, 65), bear out this latter view. The dip in the growth curve, while possibly accented by a latent or "eclipse" phase (67), most likely is a reflection of superimposed concurrent death and growth curves (15). This is supported by the rate of die-off of residual eastern encephalitis virus in the gut of *C. quinquefasciatus*, a refractory mosquito; the rate is about equal to that appearing to occur in susceptible vectors after ingestion of virus in high titer.

Direct proof of virus multiplication is dependent upon recovery of significantly greater amounts of virus from the mosquitoes after incubation than was originally ingested. This accomplishment has been no problem with the viruses that are infectious in low concentration for their susceptible vectors. Over a millionfold increase of St. Louis encephalitis virus in *C. tarsalis* has been demonstrated (18), and over a thousandfold increase of Japanese B encephalitis virus, in *C. tritaeniorhynchus* (43) and *C. quinquefasciatus* (65). Thomas (85) and Barnett (2) have recovered more western encephalitis virus from *C. tarsalis* than was ingested, and Chamberlain & Sudia (23) have reported a 10,000-fold increase in this same species.

Because of generally higher infection thresholds, eastern encephalitis multiplication has been less easily shown, but Merrill, Lacaillade & Ten-Broeck (68) obtained increases of 1000- to 10,000-fold in *A. aegypti* and *Aedes sollicitans* (Walker), and Sudia *et al.* (84), a 500-fold increase in *A. sollicitans*. Ten thousand times as much Venezuelan equine encephalomyelitis virus as ingested was recovered from *A. triseriatus* (17).

It is of interest that, in spite of considerable virus growth in mosquito tissues, no evidence of pathology has yet been found. Numerous investigators attest to the fact that infected and non-infected mosquitoes survive equally long. LaMotte (65) failed to show a difference in longevity between normal *C. quinquefasciatus* and that mosquito infected with Japanese B encephalitis virus. Thomas (85) also could not reveal differences between normal and western encephalitis-infected *C. tarsalis;* nor have cytological techniques revealed evidence of damage (65). The relationship of the mosquito with the mosquito-borne viruses is apparently highly compatible.

Undoubtedly, virus multiplies rapidly in mosquitoes following ingestion. Within four to six days certain mosquitoes have been shown to contain about as much virus as they ever will for the remainder of their life span [eastern, western, and St. Louis encephalitis (15, 22, 26); western encephalitis (85); Murray Valley encephalitis (67); West Nile encephalitis (55); Japanese B encephalitis (43)]. The actual rate of multiplication following infection by feeding is often difficult to determine, however, since residual virus in the partially digested infected blood meal may obscure the detection of new virus. Closer estimations of initial virus growth rates have been obtained by inoculation of low concentrations of virus directly through the thoracic wall of the mosquito into the body cavity. Such studies with eastern encephalitis virus in *A. triseriatus* (25) revealed an apparent tenfold increase of virus every eight hours. About eight to 10 eight-hour periods were required for an inoculum of 0.3 mouse $LD_{50}$ unit of virus to reach peak levels of approximately 1,000,000 to 2,000,000 $LD_{50}$ per mosquito. When 300 mouse $LD_{50}$ units were inoculated, only about five eight-hour periods were required for attainment of this same peak level. A nearly straight-line relationship between rates of virus multiplication in the dilute and concentrated inocula groups suggested that no appreciable lag period followed inoculation. No clear evidence of stepwise growth was observed.

With inoculation of St. Louis encephalitis virus into *C. pipiens* and *C. quinquefasciatus* [Sudia (83)], an approximate ten- to thirtyfold increase every 12 hours occurred during the period of most rapid growth until a peak of 1,000,000 $LD_{50}$ had been reached. There appeared to be a lag period in the initial growth stages, however, as indicated by the 48 to 60 hours required for an inoculated 0.7 mouse $LD_{50}$ to increase to 12 $LD_{50}$ (the lowest detection level in a triturated mosquito by the laboratory methods used).

McLean (67) produced evidence that Murray Valley encephalitis virus increased at different rates in various tissues of *Culex annulirostris* Skuse infected by intrathoracic inoculation. It grew most rapidly in the thoracic tissue, followed by the salivary glands and, lastly, the gut. Sudia (83) also found indications of differential growth rates of St. Louis encephalitis virus in tissues of *C. quinquefasciatus*. When 700 $LD_{50}$ of the virus were inoculated intrathoracicly, a plateau in the growth curve occurred between 36 and 48 hours of incubation, followed by resumption of the original rate of growth; when only 0.7 $LD_{50}$ were inoculated, a similar plateau occurred. but between

96 and 120 hours. Possibly the first part of the growth curve in each instance reflected rapid increase of virus in some tissues but not others, and the plateau indicated the virus maintenance or equilibrium level in these tissues. Then virus growth in other tissues, perhaps the salivary glands, at first lagging behind, may have overtaken and surpassed the first.

*Virus distribution.*—Although only a few days are required after virus ingestion for high concentrations to be attained in the mosquito, two to four times that incubation period is generally needed before efficient transmission by bite can occur. The work of Whitman (92) indicates that a shifting of virus infection sites occurs as incubation progresses, with an increase of concentration in the salivary glands and a decrease of amounts elsewhere in the mosquito body, perhaps caused by a depletion of susceptible cells in the sites first infected. He found that during early incubation more yellow fever virus was present in the mid-gut than in the salivary glands of *A. aegypti* but that later the reverse was true.

The temperature of incubation strongly affects the development of infection in the salivary glands. Since mosquitoes are cold-blooded animals and rates of virus growth appear to be influenced strongly by the metabolic activity of the host, a reduction in virus growth rate at decreased temperatures is to be expected. At hibernation temperatures, growth essentially stops (64). With a less severe reduction in incubation temperature (a 10° drop from 80° to 70°F.), the depression in virus growth rate is not so readily apparent; grinding and titration of individual *A. triseriatus* infected with eastern encephalitis at different incubation periods revealed nearly identical log titers of virus in the 80° and 70°F. groups. However, a doubling of the time required to achieve peak transmissibility (34 days at 70° rather than 17 days at 80°F.) indicated that the 10° drop in temperature had actually halved the virus growth and distribution rate (22).

Chamberlain *et al.* (26), working with St. Louis encephalitis virus in *C. quinquefasciatus* and *C. pipiens*, found that, within a particular homogeneous group of infected mosquitoes incubated equally long, those containing the most virus generally transmitted with highest efficiency. Probably, high levels of virus in the mosquito tissues allowed more to spill out into the haemolymph, causing greater exposure of the salivary glands to infection, which in turn resulted in an increased transmission rate. In a similar vein, mosquito infection established from ingestion of virus in concentration considerably above the threshold level produced a higher proportion of transmitters among the infected specimens than did infection from ingestion of virus in bare threshold amounts. Apparently, a longer growth period is required if virus multiplication gets off to a slow start because of single, rather than multiple, gut cell infection.

When infection of the mosquito is by intrathoracic inoculation of virus, even in small amounts, rather than by ingestion, the extrinsic incubation period may be greatly shortened. This has been shown in studies on infection of *C. pipiens* with St. Louis encephalitis virus by feeding (26) and by inocula-

tion (83). When infection was by feeding, the *C. pipiens* required 12 days of incubation for all the mosquitoes to become capable of transmitting, whereas only six days were required when virus was inoculated. Thus, by-passing the gut established 100 per cent infection of the salivary glands in about half the time. These results suggest that, when infection was by ingestion, virus was held in gut tissue for a period of up to six days before being released into the haemolymph for distribution.

Apparently, the "gut lag period" varies considerably between individual mosquitoes, since a few (7 per cent) were able to transmit as early as five days after virus ingestion. If we assume the gut lag period of these few "fast" individuals to have been negligible, then these five days represent the incubation time required in tissues other than gut before effective transmission by bite could occur. This calculated extra-gut time period is equivalent to that determined by the intrathoracic inoculation method.

Evidence is good that the salivary glands are infected by way of the haemolymph. Infection of the glands by injection of even small amounts of virus into the haemocoele suggests that infected haemolymph is the natural means of virus dissemination throughout the mosquito body. Many elaborate and tedious dissection studies verify this view. Davis & Shannon (32) dissected *A. aegypti* infected with yellow fever virus and subsequently found the virus in all tissues examined, including the body fluids. Whitman (92) found yellow fever virus first in the mid-gut and later in all parts of the mosquito, and McLean (67) revealed the presence of Murray Valley encephalitis virus in the gut, thorax, legs, and salivary glands of *C. annulirostris* at various periods after ingestion. LaMotte (65), in the most detailed dissection study of all, worked with Japanese B encephalitis virus in *C. pipiens* and found first a concentration of virus in the abdominal mid-gut, then in the haemolymph, foregut and diverticula, cephalic and thoracic ganglia, ovaries, and salivary glands. In view of the high concentrations of virus occurring in the ganglia, he pointed out the possibility of virus transport from the gut to the salivary glands by way of the nerves (64). In later judgment, however, he felt that infection of nervous tissue was merely a reflection of the broad neurotropic affinities of the virus in both vertebrates and mosquitoes and that a more tenable manner of salivary gland infection was by way of the insect blood (65). In fact, his studies prove the presence of a viremia from the second week to about the end of the fourth. The viremia was frequently high during this period but became progressively lower during the fourth week. Apparently, viremia production, as well as neurotropism, in both vertebrates and mosquitoes is a basic property of these viruses.

Recent experiments of a different type also indicate the haemolymph route of salivary gland infection (21). When eastern encephalitis-immune serum was inoculated into the haemocoele of *A. triseriatus* and an infected blood meal given two days later, the 17-day transmission rate was reduced from 68 per cent (for controls inoculated with normal serum) to only 19 per cent. This can be taken as evidence of decided blockage of salivary gland in-

fection. When the infected blood meal was given first and the immune serum was not inoculated until four days later, the transmission rate at 17 days of incubation was unaffected, indicating that virus had already been released from the gut tissue into the haemolymph to establish salivary gland infection prior to the immune serum inoculation. Similar results were obtained with *C. quinquefasciatus*, using St. Louis encephalitis virus and its immune serum. The mosquitoes were infected by feeding upon a viremic chick and then inoculated at either two or five days of incubation with immune serum of St. Louis encephalitis. On the fifteenth day of incubation, the transmission rate of the two-day group was only 6 per cent versus 61 per cent for controls; that of the five-day group was depressed a lesser amount, 47 per cent versus 78 per cent. These findings suggest that virus had not yet been released into the insect blood from the gut tissue on the second day but had in a considerable proportion of the mosquitoes by the fifth day.

These same studies also furnished strong evidence that virus actually multiplies in the salivary glands and does not merely accumulate there. If infection of the salivary glands was by means of virus accumulation rather than multiplication, one would have expected the inoculation of eastern encephalitis-immune serum on the fourth day following an infected blood meal to have depressed the transmission rate by reducing the amount of virus in the haemolymph. Rather, the results indicated actual salivary gland infection prior to the inoculation of the immune serum. Similar indications are found in the results of the inoculation of immune serum of St. Louis encephalitis on the fifth post-infection day.

*Salivary gland infection and transmission by bite.*—Mosquito infection rates of 100 per cent are common in the laboratory and can be expected in almost any susceptible vector species provided the virus meal is well above the threshold level and the incubation period is adequate. On the other hand, transmission rates at or near 100 per cent are rarely encountered. They have been demonstrated only with St. Louis encephalitis virus in *C. tarsalis*, *C. quinquefasciatus*, and *C. pipiens* (23, 26), Japanese B encephalitis virus in *C. quinquefasciatus*, *Culex molestus* Forskål, and *C. tarsalis* (65), and Venezuelan equine encephalomyelitis virus in *A. triseriatus* (13, 17). In other mosquito species infected with these and numerous other viruses, transmitting efficiency has been appreciably less. For example, with infection rates all at or near 100 per cent, only 39 per cent of *A. sollicitans* transmitted western encephalitis virus (20); 52 per cent of *A. triseriatus* transmitted Japanese B encephalitis virus (65); and 16 per cent of *Aedes dorsalis* (Meigen) transmitted St. Louis encephalitis virus (26). An extreme example is found in *Anopheles quadrimaculatus* Say and eastern encephalitis virus; although 79 per cent of the individuals contained considerable amounts of virus in their bodies, none was capable of transmitting (20).

Failure to transmit to a susceptible host is obviously a sign that an infectious quantity of virus was not injected into that host during feeding. This could be attributed to the absence of gland infection. However, a mosquito

with infected glands might still fail to transmit if sufficient saliva were not injected during the feeding act or if the infection in the glands were of low level, with little virus in the saliva. Thomas (85) has presented evidence that the rate of transmission of western encephalitis virus varies directly with the concentration in the salivary glands.

Absence of salivary gland infection might be caused by failure of virus to reach the salivary glands or, once reaching them, being unable to penetrate the glandular tissue to establish infection. An inadequate viremia in the mosquito despite substantial virus growth in the gut could account for an unsuccessful gland infection just as certainly as insusceptibility of the glands themselves. An approach to clarify this point would be to determine the transmitting ability of mosquitoes infected by intrathoracic inoculation of minimal amounts of virus. If they failed to transmit, a barrier in the salivary glands themselves would be indicated. If they did transmit following inoculation, but not after infection by feeding, despite gut infection, poor virus transfer from gut to glands would seem the more logical explanation. Unfortunately, little work of this type has been done. Although all the biologically transmitted mosquito-borne viruses thus far tested have infected any mosquito species into which they have been inoculated, few of the species have been checked subsequently for their ability to transmit.

Whatever the mechanism of the barrier to salivary gland infection, its presence or absence, together with the threshold of gut infection, is of primary concern in determining the epidemiological importance of a vector species. Other factors being equal, those mosquitoes easily infected and transmitting with high efficiency will be most apt to pick up and transfer virus from host to host.

In mosquitoes with established salivary gland infections, variations in the feeding technique of different species and of individuals within a species undoubtedly affect the transmitting efficiency. For instance, some species probe much more actively than others. It seems probable that a larger injection of saliva is made at the initial stage of feeding than after a satisfactory blood supply is tapped. Probing alone, without completion of the blood meal, appears to result in as efficient a transmission as does taking a complete meal (21). Also, on occasion, several transmissions in a row have been accomplished by inducing an infected mosquito to probe first upon one host, then another (13, 21).

Several studies have revealed a decreasing ability to transmit in mosquitoes after lengthy periods of incubation (15, 22, 23). General weakness, with ineffectual salivary injection, may be accountable, or, perhaps, a decreased concentration of virus in the glands because of cessation of virus multiplication may be the cause.

The amount of virus injected by a mosquito during feeding is highly variable. Davis (30) estimated that *A. aegypti* inoculates approximately 100 MLD of yellow fever virus; Chamberlain *et al.* (16) estimated that more than 10,000 mouse $LD_{50}$ of eastern encephalitis virus could occasionally be

injected by this same species, although most individuals inoculated much lesser amounts. The extremely high and uniform transmitting efficiency of St. Louis and Japanese B encephalitis viruses by certain *Culex* species suggests inoculation of relatively large amounts of virus (26, 65). This great efficiency is most probably attributable to a high concentration of virus in the salivary secretions.

*Persistence of virus in mosquitoes.*—Once infected, a mosquito generally remains infected for its entire life. This is common knowledge among those who have worked with infected mosquitoes in the laboratory. The earliest of many studies on this subject go back to Bauer & Hudson (5), who produced yellow fever infection by the bite of a single mosquito 91 days after feeding upon an infected monkey. More recently, field evidence has indicated that yellow fever virus may possibly survive a tropical dry season in long-lived *Haemagogus mesodentatus* Komp & Kumm, *Haemagogus equinus* Theobald, and *Sabethes chloropterus* (Humboldt) (9, 40, 86).

Experiments conducted under seminatural and simulated winter conditions have also shown long survival of virus in mosquitoes. Bellamy *et al.* (6) held western encephalitis-infected *C. tarsalis* in a cellar in Kern County, California, under natural winter temperatures three to four months and found that some were still able to transmit by bite. *C. quinquefasciatus* infected with St. Louis encephalitis virus and held under the same conditions survived and remained infected for up to 116 days. They found some evidence, however, that western encephalitis virus may become attenuated after long low-temperature incubation of its vector. Hurlbut (52) showed that *C. quinquefasciatus* infected with Japanese B encephalitis incubated first for 15 days at 20°–30°C., then 82 days at 8°–13°, followed by three days at 30° were still able to transmit by bite. LaMotte (64) confirmed the work of Hurlbut and showed that, although virus was apparently lost from some of the mosquitoes incubated first for three days at normal temperatures and then given prolonged exposure to 10°C., others remained infected for at least two months. When these mosquitoes were subsequently warmed up to 26.5°C., the virus in those still infected proceeded to increase at a rate similar to that expected had the low temperature not been interposed.

The epidemiological significance of persistent virus infections in mosquitoes is easily grasped. In favorable weather, a transmission season as long as the life span of the mosquito is assured. Furthermore, lifelong infection of true hibernating mosquitoes may explain, at least in part, the overwintering of virus in colder temperate regions where continuous transmission is interrupted. Supportive evidence of this overwintering mechanism is found in an isolation of western encephalitis virus from hibernating *C. tarsalis* in a Colorado mine in late December (8). Contradictory evidence is supplied by Bennington *et al.* (7), however, who showed that *C. tarsalis* in Colorado requires a carbohydrate rather than a blood meal for proper development of fat body for successful hibernation. Similarly, Wallis (89) found that *Culex restuans* Theobald has the same requirement in Connecticut. In view of the

importance of determining the mechanism of overwintering of mosquito-borne viruses in temperate regions, further studies on the nutritional requirements of various mosquito species for hibernation are indicated and should receive a high priority.

Persistence of virus in the mosquito may be explained by continuous multiplication with die-off equal to, or only slightly exceeding, replacement rate or by exceptionally efficient stabilization of virus by the insect tissues. While an equilibrium between growth and death at first appears most likely, a good case can be presented for stabilization. Kissling (59) has shown that eastern encephalitis virus diluted in 50 per cent normal horse or guinea pig serum or in 20 per cent yolk of seven-day-old embryonated eggs can be left at room temperature 25 to 35 days without a drop in titer. Nearly as remarkable stabilization occurred with western and St. Louis encephalitis viruses. Possibly, mosquito haemolymph could provide similar efficient stabilization; however, such stabilization would probably not last the entire life of the mosquito, as indicated by the findings of LaMotte (65) that Japanese B encephalitis virus in *C. pipiens* was mostly gone from the haemolymph by the end of the fourth week. Probably, lifelong virus persistence is best explained by combined action: a long period of progressive virus multiplication in various organs together with a slow virus die-off.

*Dual infections.*—The possibility of naturally occurring infections of mosquitoes with two different viruses at the same time has intrigued a number of workers. More than one virus has, on occasion, been isolated from a single mosquito pool, but, since the usual procedure is to grind a number of individuals in a single pool for testing, such a mixed infection does not necessarily mean that both viruses came from the same mosquito. Quite to the contrary, chances are strongly against it. Assuredly, isolation of a mixture of two viruses from a single arthropod pool can be confusing, as illustrated by the experience of Hammon *et al.* (46); they seriously considered the possibility that a mixed infection of western and St. Louis encephalitis viruses was actually a single stem-virus showing characteristics of both.

Sabin (72) attempted dual infection of *A. aegypti* with the viruses of dengue and yellow fever. His results indicated that prior dengue infection made the mosquitoes relatively less susceptible to yellow fever virus. For instance, some of the dengue-infected mosquitoes did not become infected with yellow fever virus, whereas all the normal mosquitoes did. Indications were that prior dengue infection raised the threshold to yellow fever infection. In summary, he suggested that this interference in mosquitoes might be a factor in explaining the absence of yellow fever from areas in the Orient where dengue is common. In view of the low field viruses infection rates generally encountered in mosquitoes, however, this possibility seems remote. Present knowledge indicates that, if prior infection with dengue is a factor, it is more likely the dengue-immune status of the human host that is important, furnishing by cross-reaction a partial protection against yellow fever infection (11).

Successful dual infection of eastern and western encephalitis viruses has been accomplished in *C. tarsalis* by first feeding the mosquitoes upon an eastern encephalitis-infected chick and then one week later upon a western encephalitis-infected chick (24). All the mosquitoes thus exposed became dually infected, with the concentration of each virus in their bodies equivalent to that in control mosquitoes fed only one virus or the other. Both viruses were transmitted concurrently on several occasions, showing that the dual infections could occur in the salivary glands as well as in other parts of the mosquito body.

Perhaps the viruses of dengue and yellow fever are more closely related than those of eastern and western encephalitis, which may account for the observed interference between them but not between eastern and western encephalitis. However, in Sabin's experiments, the amounts of virus given in the mosquito meals were apparently near threshold levels, allowing effects upon threshold to show up more clearly. In the eastern and western encephalitis experiments, the concentrations of virus fed upon were considerably in excess of threshold levels, and minor differences could have been obscured.

Despite relative distinctness of eastern and western encephalitis viruses serologically, they still appear to be closely related (11). They infect the same vertebrate and mosquito hosts in the laboratory, and neither cause detectable cell changes in the mosquito nor do they adversely affect its activity or life span. In view of their similarity of action, affinities for different tissues and cells seem unlikely. More likely, they develop similarly in their mosquito hosts and infect the same kinds of cells. Presence of both viruses in the salivary glands, organs of restricted cell types, suggests this to be the case.

Dual infection of the same individual cells might occur. However, this would require separate enzyme systems within the cell or joint use of the same enzyme system, and, as a consequence, one would expect reduced virus yield because of competition for metabolic substrate and perhaps even production of hybrid forms; neither of these reactions appears to have taken place. A more probable explanation for apparent lack of interference is that both eastern and western encephalitis viruses grew in the same types of cells but that only a portion of the total number of cells were infected, leaving plenty of room for both viruses to grow concurrently and separately. This hypothesis suggests that the mosquito possesses a defense mechanism to limit virus infections, otherwise all susceptible cells would soon become infected, even with single infections. Waste products of virus multiplication itself might serve such a function.

*Transovarian transmission.*—Transovarian passage of virus from one generation of mosquito to the next is one of many mechanisms being considered as a means of virus overwintering in temperate zones. Thus far, however, the evidence for effective transovarian transmission is contradictory and incomplete.

On the negative side, early reports of transovarian passage of Japanese B encephalitis virus in mosquitoes (70) could not be confirmed and were later retracted (69). Although isolations of eastern encephalitis and Venezuelan

equine encephalomyelitis viruses have been made from eggs of infected *Mansonia perturbans* (Walker) (19) and of western encephalitis virus from those of *A. triseriatus* (63) and *C. tarsalis* (85), none of these eggs was hatched and a second generation of infected adults obtained. Attempts to isolate Venezuelan equine encephalomyelitis virus from the eggs of *A. triseriatus* were negative (17, 61). Thousands of adult mosquitoes reared from eggs of *C. quinquefasciatus* infected with St. Louis encephalitis virus were found to be virus negative (26), and several attempts to isolate western encephalitis virus from progeny of infected *C. tarsalis* met with failure (2, 23, 44). Attempts to prove transovarian transmission of West Nile encephalitis virus by infected *A. aegypti* also failed (28).

On the more positive side, Whitman & Antunes (94) have shown that *A. aegypti* larvae immersed in a suspension of yellow fever virus can become infected and carry this infection into the adult stage, and the larvae of *C. tarsalis* have been similarly infected with western encephalitis virus by Thomas (85). Thus, the necessary intermediate stages of mosquitoes have been proven to be susceptible. Encouraging also is a recent report by Wu & Wu (96) that seven isolations of Japanese B encephalitis virus were made from adult *Aedes albopictus* (Skuse) reared from larvae breeding near houses of people infected with this virus. Furthermore, Daniels (27) has made an isolation of eastern encephalitis virus from field-collected larvae of *Culiseta melanura* (Coquillett) in Massachusetts, and another isolation of this virus was made from males of *C. melanura* collected in southern Alabama (21). In the latter instance, however, infected females were taken in the same trap collection, leaving the possibility that virus contamination of the males might be accountable.

Recent findings of LaMotte (65) may be of utmost significance in respect to the transovarian question. He has shown that the ovaries of *C. pipiens* infected with Japanese B encephalitis did not contain virus in appreciable quantity until the second week of incubation; the virus concentration reached a peak during the third week. Likewise, Thomas (85) found a greater concentration of western encephalitis virus in the ovaries of *C. tarsalis* on the tenth day than on the fourth. These data cast considerable doubt on the reliability of experimental procedures heretofore used in testing the ability of mosquitoes to transmit by the transovarian route. Obviously, collecting the first eggs laid by a female following the infected blood meal, at a time when ovarian infection is minimal or absent, is not a true measure of its ability to transmit by the ovarian route. As LaMotte suggests, eggs from a second blood meal, given at a time when considerable virus is associated with ovarian tissue, would be more likely to furnish positive results. Certainly, the possibility of transovarian passage of mosquito-borne viruses needs further checking and evaluation.

At this point, the most important phases of virus infection of mosquitoes, excluding ecological factors, have been considered, discussed, and to some degree evaluated. It is obvious that, while progress has been significant, a considerable amount of research still remains to be done.

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# ARTHROPOD VECTORS AS RESERVOIRS OF MICROBIAL DISEASE AGENTS[1]

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The subject of arthropod vectors as reservoirs of agents of animal diseases is difficult to review because of divergent viewpoints of the definition of a "reservoir." Some investigators would reserve this term for vertebrate hosts and the term "vector" for intermediate hosts (141). Others would consider vectors to be reservoirs if they participate in an essential phase of the disease cycle, or show cyclic propagation or transovarial transmission of the pathogen [a comprehensive, tabular review of ticks in this role is supplied by Neitz (126)]. Other factors also complicate precise definition of the word "reservoir." These include: (*a*) length of life and types of metamorphosis of the arthropods; (*b*) method of transmission by biting or by contamination from fresh or old feces; and (*c*) "premunition" (latent persistence of organisms after initial infection) in the vertebrate host and the too often speculative role this plays in providing a source of infection for ectoparasites.

In this review, the reservoir mechanism is envisioned as a flexible biological adjustment of the vertebrate and arthropod hosts to pathogens, in which the relative importance of either host as a reservoir varies in different diseases. Space permits only restricted selection of evidence that emphasizes the role of arthropods beyond that of simply being vectors (e.g., yellow fever virus and mosquitoes). The terms "transovarial" (or "hereditary") and "transstadial" refer to passage of disease agents through ova and between active stages of a given vector, respectively.

## Virus Diseases

There are well over 70 viruses that are known or suspected to be arthropod-borne, and new isolations, especially from mosquitoes, are continuously being reported. For the present review, insect- and acarid-borne virus diseases are treated separately, but only those having some arthropod-reservoir implications are considered. For a detailed discussion of these arthropod-borne viruses, refer to Rivers & Horsfal (163).

*Mosquito-borne virus diseases.*—In the tropics, long-term reservoirs may not be necessary because of continuous exchange of virus between susceptible hosts and vectors. Under more temperate conditions, however, where vector populations become dormant or hibernate, certain reservoir mechanisms are required for survival of viruses during the winter as well as between out-

[1] The survey of the literature pertaining to this review was concluded in March, 1960.

breaks of disease. Because of repeated isolations of St. Louis encephalitis and western and eastern equine encephalitis viruses from mites, bugs, and other ectoparasites [reviewed by Eklund (65, 66) and Miles (119)], it has been postulated that arthropods other than mosquitoes may play a role in maintaining these viruses in nature. However, experimental studies to determine the significance of such arthropods in maintenance of virus have clearly demonstrated that virus survives in them for a relatively short period only and is seldom transmitted during the feeding process (34). Thus, such arthropod virus isolations may merely represent a concomitant infection of their host animals. Recent reported isolations of eastern equine encephalitis (first reported as western equine encephalitis) from naturally infected *Ixodes ricinus* Linnaeus in Czechoslovakia (111) renewed interest in the experimental findings of transstadial and transovarial virus infection of western equine encephalitis in *Dermacentor andersoni* Stiles (189), of St. Louis encephalitis in *Dermacentor variabilis* (Say) (16), and of Japanese B encephalitis in several species of Asiatic ixodid ticks (39). In spite of the failure of *D. variabilis* to transmit virus of western equine encephalitis (83) and the report of Benda & Danes (12) of survival of eastern equine encephalitis in *I. ricinus* for four weeks only, the question of whether ticks play a part in the maintenance of these viruses deserves further investigation inasmuch as many species of birds and rodents, regarded as donors, are parasitized by ticks. In this respect, the data on maintenance and transmission of West Nile fever virus by experimentally infected *Ornithodoros coniceps* (Canestrini) reported by Vermeil *et al.* (198) may be pertinent. They considered this tick, a common parasite of birds and particularly pigeons, a potential reservoir of West Nile fever virus in nature.

Recent virus studies by Smorodintsev & Drobyshevskaya (181) and Buescher *et al.* (20) with Japanese B encephalitis, Barnett (7) with western equine encephalitis, and Chamberlain and associates (35, 36) with western and eastern equine and St. Louis encephalitis gave no evidence of transovarial passage of the viruses in their respective mosquito vectors.

Until the findings of transovarial passage of Japanese B encephalitis virus reported by Wu & Wu (211) and Kissling *et al.* (99) have been further studied, it must be assumed that passage of virus to the progeny via eggs of infected mosquitoes is a doubtful reservoir factor.

Occasional isolations of western equine encephalitis from hibernating *Culex tarsalis* Coquillett (159) and results of studies by Bellamy *et al.* (10) suggest that overwintering mosquitoes may be capable of preserving infection for more than short periods of time. However, observations on the behavior of Japanese B encephalitis virus in experimentally infected bats (106), isolations of western equine encephalitis from birds with latent infections (160), and the discovery of the susceptibility of snakes to the latter virus (192) suggest that vertebrates may also be involved in overwintering mechanisms. A combination of several of these factors therefore may be responsible for the recurrent appearance of virus in endemic areas.

*Tick-borne virus diseases.*—In contrast to mosquitoes, ticks have been shown to be both vectors and reservoirs of various viruses. They can remain infectious for long periods of time (including over winter), transmit effectively in immature and adult stages, and often pass the viral agents to their progeny. For example, *Ixodes persulcatus* Schulze is the most important reservoir of Russian spring-summer encephalitis virus in nature because of the ticks' ability to preserve the virus throughout the winter and to transmit it transovarially to their progeny (40, 174). *Dermacentor silvarum* Olenev and *Haemaphysalis concinna* Koch have been found naturally infected (166, 167), but, according to Smorodintsev (177, 179), the importance of these ticks as natural vectors of Russian spring-summer encephalitis is still undetermined, since virus does not persist in them longer than 30 days. *I. ricinus*, the vector of louping-ill, biundulant meningoencephalitis, and of several other reported central European tick-borne encephalitides, has been shown to be a lifelong carrier of Russian spring-summer encephalitis virus (15). This tick transmits louping-ill virus transstadially only (113), but transovarial passage has been claimed for the viruses of Russian and central European encephalitides (11, 38, 86). It appears, however, that transovarial transmission is not as common as in *I. persulcatus* (11). There are no data available on infection rates in the progeny, but, based on the infrequent virus isolations from ticks collected in encephalitis foci (108, 120, 166, 213), the percentages of transovarially infected ticks appear rather small. Whether specific encephalitis antibodies present in immune host animals have an effect on the infectiousness of ticks is a question requiring clarification. Some investigators believe that transstadial and transovarial transmission is entirely impeded (133). Others observe a considerable reduction in virus amounts (11, 107), while still others find no such effect (64).

The discovery of other tick-borne encephalitides immunologically related to Russian spring-summer encephalitis suggests that the viruses of this complex may be transmitted by a variety of ixodid ticks in different localities. Thus, in the viruses of the Soviet tick-borne hemorrhagic fevers, the vectors are considered to be also interepidemic reservoirs of the agents in nature (37, 180). In India, isolations of Kyasanur Forest Disease virus from *Haemaphysalis* ticks, particularly *H. spinigera* Neumann, indicate that this species may be involved in virus maintenance (195). A virus similar or identical to Russian spring-summer encephalitis has been isolated from *Ixodes granulatus* Supino taken off Malayan forest rats (176).

So far as is known, Colorado tick fever is the only tick-borne virus disease in the United States, but, probably, it is the most prevalent of any human tick-borne disease in the West. Although virus has been isolated from several western tick species, *D. andersoni* is the only known vector to man (68). Virus is maintained in a seasonal cycle between immature ticks and a variety of small vertebrates, mostly rodents (27). Overwintering nymphal ticks infect susceptible rodents, which develop an extremely long viremia (up to 55 days) (24, 26). Occurrence of transovarial transmission, reported

by Florio *et al.* (71), has not been confirmed, and, if it does occur in nature, it must be extremely uncommon (67).

Nairobi sheep disease virus was the first to be shown to be transmitted transovarially by its tick vector, *Rhipicephalus appendiculatus* Neumann (43). This virus was found to disappear in the salivary glands, probably during the molting process of the tick, following an infective feed on a susceptible or an immune animal (43). Laboratory studies indicated long survival of the virus in this tick. According to Lewis (109), unfed larvae retain the virus for 245 days, unfed nymphs for 359 days, and unfed adults for as long as 871 days.

*Phlebotomus fever.*—As early as 1909, Doerr & Russ (62) suggested that sandfly fever is maintained in nature by a multiple relationship in which the vector, *Phlebotomus papatasi* Scopoli, plays an important role because of its ability to transmit the causative agent transovarially.

Transmission by flies that had no previous access to any source of virus was reported by Whittingham (203). He believes, however, that these flies became infected by feeding on dejecta or dead bodies of infected adult flies rather than by transovarial passage of the virus. Young *et al.* (212) considered mites that parasitize adult flies to be the true reservoir; this is difficult to accept, since Moshkovsky *et al.* (123) produced phlebotomus fever in persons bitten by flies raised under mite-free conditions. Sabin *et al.* (168) were unable to demonstrate infection in the offspring of infectious flies and concluded that transovarial infection is not regular. The questions of how virus is maintained through the winter and what is the reservoir in nature are not as yet settled.

## Rickettsioses

The term "rickettsioses" is retained for diseases in animals, including man, caused by arthropod-borne rickettsiae and rickettsia-like organisms in several genera, most of which were originally described in *Rickettsia* (*sensu lato*). The group furnishes perhaps the best examples of vertebrate pathogens that appear to have originated in arthropods in a primitive symbiotic association (184). Many of these organisms also produce latent infections in vertebrate hosts (145). About as many of these organisms are known to occur only in arthropod hosts as are considered to be pathogens for vertebrates. In contrast, no typical rickettsiae are confined to vertebrate hosts alone, although the Q fever organism in man and domestic animals appears to have little dependence upon invertebrates. It is not difficult to accept arthropod hosts as supplemental or major reservoirs of rickettsiae.

Two startling developments in the past decade implicate large domestic animals in local cycles of epidemic typhus in Abyssinia and of Rocky Mountain spotted fever in northern Mexico. (*a*) *Rickettsia prowazekii* da Rocha Lima was recovered by Reiss-Gutfreund (161) from one goat, two sheep, one patient, and one tick species each of *Hyalomma* and *Amblyomma* collected from cattle and camels. The isolates had classic pathogenic and serologic

characteristics and were transmissible through rabbits to human body lice and to immature ticks of four species. Approximately a fourth of the serums of the nearly 1000 cattle, sheep, and goats examined agglutinated the rickettsia at a titer of 1:640 or more. She concludes that, under the local conditions, certain domestic animals and their parasites could be true reservoirs of typhus fever. Identification of the organisms as *R. prowazekii* was confirmed in Paris. (*b*) Silva-Goytia and colleagues (175) found unsuspected ticks, *Rhipicephalus sanguineus* (Latreille), *Ornithodoros nicollei* (Mooser), and *Otobius lagophilus* (Cooley & Kohls), to be infected with spotted fever and observed that livestock on the premises where human cases had occurred had higher complement-fixing antibody titers than did animals in non-focal areas.

*Typhus.*—Since lice are too short-lived to serve as the reservoir of epidemic typhus, man has been considered to perform this function. The studies outlined in (*a*) above suggest that, under certain conditions, there may be a reservoir cycle in addition to the classic man-louse-man one and open new problems for investigation. Rats and their ectoparasites also have been accused of participation in the cycle (93, 100).

The long persistence of *Rickettsia typhi* (Wolbach & Todd) in tissues of commensal rats (141) makes it difficult to visualize the customary flea vectors as more than essential intermediates in transmission of endemic typhus. In spite of their relative inefficiency as experimental vectors, certain dermanyssid mites could, through frequent feedings (as in rickettsialpox), aid in maintaining infection among rats in nests. Adult *Ornithonyssus bacoti* (Hirst) transmitted the agent to rats after a seven-day incubation period, and through eggs to immature stages of the next generation (63); however, Worth & Rickard (210) failed to confirm this. Naturally infected mites have been found on several occasions in China (132). In isolated instances, *R. typhi* has been recovered from two species of rat fleas in Galveston, Texas (186), and from two pools of rat fleas, as well as the tick, *Boophilus* sp. (*"australis"* Fuller), off bushes near a military camp in India where a clinically diagnosed case had occurred eight days previously in an individual who had been bitten by a tick (95). In Java, Gispen (80) found five lots of larval trombiculid mites, *Euschöngastia indica* (Hirst), from suburban house and sewer rats to be infected. Since the hosts of four of these lots were uninfected, this convinced him these larvae (non-parasitic as nymphs and adults) had received the infection from the previous generation. In Manila, this mite was a more common parasite than fleas and occurred in the ears of commensal rats, 18 per cent of which had murine typhus antibodies (209).

*Tick-borne rickettsioses.*—In Rocky Mountain spotted fever, the short duration of circulating *Rickettsia rickettsii* (Wolbach) in susceptible small animal hosts places emphasis on ticks as reservoirs. In addition to different host habits of various vectors, three factors operate in the maintenance of infection by ticks: (*a*) transovarial transmission, (*b*) new lines of infection by simultaneous feeding of infected and non-infected ticks on susceptible hosts, and (*c*) infection of tick ova by rickettsiae-bearing sperm during copulation

(146). With the increasing number of rickettsial agents that do not show the unique change to an avirulent phase attributed to *R. rickettsii* in fasting ticks, this phenomenon should be re-examined quantitatively. Price (155) apparently confirmed the qualitative aspects of this phase of the reservoir mechanism; it is hoped that fluorescent antibody techniques will provide a new tool for critical re-evaluation of the question. Different workers have shown that certain levels of virulence of rickettsiae are maintained in different ticks as well as in various foci. There are unknown factors that keep the level of virulence low in *Haemaphysalis leporis-palustris* (Packard) (134, 155) or predominantly high on one side of the Bitterroot Valley of western Montana compared to the other (146).

The claim (114) that bed bugs are vectors in Brazil has never been substantiated; Weyer (201) was unable to maintain *R. rickettsii* in artificially infected bugs longer than 24 days.

The specificity of *Rickettsia conorii* Brumpt of the fièvre boutonneuse group (South African tick-bite fever, Kenya, Crimean, and Indian tick typhuses) has been confirmed in mouse toxin tests (8). In the Mediterranean Littoral (100), Crimea (152), and Kashmir (144), the main vector is *R. sanguineus*, whereas, in Central and South Africa, other ticks, native animals, and environments are involved (100, 220). Nevertheless, as in spotted fever, in which both transstadial and transovarial transmissions occur, ticks are the important reservoirs.

Recent unusual tick-infection records include isolations from *Boophilus* in Italy (169), *R. appendiculatus* in Eritrea (172), *Rickettsia simus* Koch in Kenya (193), *Haemaphysalis leachi* (Audouin) in Manipur State, eastern India (18), and *Hyalomma marginatum* Koch in Crimea (151).

*India.*—A disease entity, eventually labelled "Indian tick typhus," was suspected long before rickettsiae were isolated from *Haemaphysalis, Ixodes*, and *Rhipicephalus* ticks in several parts of India (94, 144). A strain from *R. sanguineus* from Kashmir was passed experimentally through eggs and active stages of two generations of that species. It could not be distinguished from tick-borne *R. conorii* (144, 215).

*Miscellaneous.*—Queensland tick typhus is still associated with the bite of *Ixodes holocyclus* Neumann through circumstantial evidence only. This evidence has been strengthened recently by Neilson (125), who studied a patient from whom a strain was isolated. As in some of the originally described cases, a primary eschar had appeared at the site from which a tick was removed eight days previously. Serological evidence suggests that there is a primary cycle between small animal hosts, particularly marsupials, and ticks, but the relative importance of vertebrate and invertebrate hosts remains to be determined. We have failed to transmit the agent, *Rickettsia australis* Philip, through the American wood tick, *D. andersoni*. Tropical rat mites retain the organisms, but only rarely do they cause infection (127).

The agent of tick-borne fever of sheep in Great Britain, *Rickettsia phagocytophila* (Foggie) (72) is transmitted transstadially but not transovarially by

*I. ricinus*. Differences from *Ehrlichia ovina* (Lestoquard & Donatien) of Africa are not stated. Since blood of sheep remains infectious for some months, ticks probably occupy a supplemental role as reservoirs.

Unnamed rickettsial agents related to *R. rickettsii* have been recovered from North American ticks, *Amblyomma maculatum* Koch (8, 105) and *Dermacentor parumapertus* Neumann (147). Ticks undoubtedly have an important role in the maintenance of these rickettsiae. Even less is known about a spotted-fever-like rickettsia reportedly isolated from *R. sanguineus* taken from asymptomatic dogs in Texas. This agent was said to be pathogenic for rabbits and guinea pigs (3).

*Northern Asia.*—In reference to Siberian tick typhus, Cox (42), following Zdrodovskiy & Golinevich (214), has recently stated: "Various species of ixodid ticks of the genera *Dermacentor* and *Haemaphysalis*, together with certain small wild animals, constitute the reservoir of the disease." The disease is widespread across Siberia south to Outer Mongolia, and to Armenia in the southwest. Six species of these ticks have been experimentally confirmed as vectors of *Rickettsia* (*Dermacentroxenus*) *siberica* Zdrodovskiy & Golinevich with proof of transovarial passage of the agent. Since there is apparently no premunition in infected rodents, ticks are considered the primary reservoirs (214). *R. siberica*, surprisingly, shows a closer relationship to "maculatum rickettsiae" and *R. rickettsii* than to *R. conorii* (8).

Another organism, *Rickettsia pavlovskii* Korshunova (102), has been said to cause a form of human "infectious nephroso-nephritis"[2] in three endemic areas in U.S.S.R., and it has been isolated from a remarkably wide variety of ectoparasites taken from various mammals. These included two species of ixodid ticks, four of gamasoid and two of trombiculid mites, and five of rodent fleas. Since strains have also been recovered from hosts of ectoparasites and from patients, the relation of arthropods in the reservoir mechanism remains to be assessed. More studies are needed, such as those showing transovarial transmission in *Ixodes laguri* Olenev, *I. ricinus* (102), and *Haemolaelaps glasgowi* Ewing (82). The mites transmitted infection to guinea pigs by bite and maintained organisms up to a year. Arthropods would appear to be important in the reservoir picture, but no report of the duration of organisms in animal tissues was found in the literature, unless the statement attributed

[2] A better known form of haemorrhagic fever of virus etiology has also been called "haemorrhagic nephroso-nephritis" (177, 180). So far, only fever therapy volunteers have proved susceptible to the agent, but infection was also found in three species of indigenous gamasoid mites which Chumakov (37) visualizes as a "virus reservoir for rodents, but they play no direct role in human infection." In contrast, the pathogenesis of *R. pavlovskii* is quite different, but, strangely, no studies on cross-immunological relationships of isolates from the various sources, including *R. siberica*, are reported. Chumakov (37) has confirmed the virus rather than rickettsial etiology of the nephroso-nephritis syndrome, but, for purposes of this section, interest remains in the biocoenose of *R. pavlovskii*, which, at present, appears to be a separate entity of uncertain relationship.

to Korshunova (214), that rickettsiae persist for 140 days in rat brains, refers to this agent.

"Tick paroxysmal rickettsiosis" is a human disease occurring in the Ukraine, characterized by trench fever-like paroxysms and absence of rash (215, 217). The "reservoir" is stated to involve *I. ricinus* and voles. Zhdanov & Korenblit (218) named the etiologic agent *Wolhynia rutchkovskii* after the investigator (Ruchkovskiy) who isolated it.

*African animal rickettsioses.*—The best known of these tick-borne rickettsioses is heart-water fever caused by *Cowdria ruminantium* (Cowdry). This disease often causes severe losses in ruminant stock and is transmitted by two species of *Amblyomma* (126). Since the organisms do not invade the body cavity of the tick but are confined to epithelial cells of the gut, passage is transstadial but not transovarial, and animals probably become infected from regurgitation of gut contents during feeding of the vector. While animals with subacute and chronic infections are undoubtedly important as reservoirs, the tick *Amblyomma hebraeum* Koch, which requires from nine months to two full years to complete its life cycle, can furnish substantial assistance in maintenance.

Three other less well-known tick-borne agents of animal disease are now assigned to the genus *Ehrlichia*, namely *E. bovis* (Donatien & Lestoquard), *E. canis* (Donatien & Lestoquard), and *E. ovina* (Lestoquard & Donatien), transmitted, respectively, by *Hyalomma* and two species of *Rhipicephalus*. *E. bovis* is reported to be transmitted transstadially in *Hyalomma*, but only *E. canis* has been shown to pass through eggs of *R. sanguineus* and to be transmitted by active stages in two generations. Since relapses occur in splenectomized dogs and blood has been found to be infective for 23 months (17), ticks can possibly become infected through feeding on dogs with latent infections. At least in the canine disease, ticks share an important part in natural maintenance.

*Mite-borne rickettsioses.*—Until recently, rickettsialpox ["varicelliform rickettsiosis" in U.S.S.R. (218)] was recognized only in urban environments where there is close association between commensal murids, *Allodermanyssus sanguineus* (Hirst), and human habitation. The disease, however, is not coextensive with known distribution of the mite (154). Cases have been confirmed in cities along the North Atlantic coast, in Cleveland, Ohio, and in the Ukraine (42). A proven isolate by Jackson *et al.* (91) from a field rodent (reed vole) in Korea suggests that there is a cycle in nature independent of man. Reports of the disease based on serological evidence alone (Bosnia and Equatorial and South Africa) is hardly acceptable, unless accompanied by isolation of the causative agent (75).

A generation of mites can be completed in 17 to 23 days under optimum conditions (74), and nymphal and adult stages of the $F_1$ generation can transmit infection after transovarial passage (98). Experimental passage through the eggs of tropical rat mites, *O. bacoti*, and transmission by bite have also been demonstrated (149). Since these mites are nest-infesting species, their

living habits increase the frequency of feedings and insure relative continuity of murid infection, thus providing an effective reservoir mechanism even though they live only a few months.

In tsutsugamushi disease (scrub typhus) of the South Pacific and Far East, the vector situation is different because the mites feed only once in a generation. Here, the ability of larval mites to serve as vectors is even more restricted than in diseases in which multiple-feeding biting flies are vectors. Though continuity of *Rickettsia tsutsugamushi* (Hayashi) in mites by transovarial transmission was axiomatic, experimental confirmation was slow; Krishnan *et al.* (104) infected five of eight mice that were bitten by 38 to 78 reared larvae of *Trombicula deliensis* Walch. It is not known if infection can persist through several generations in the absence of susceptible hosts. A fact often overlooked is that trombiculids feed not on blood but on lymph and tissues of the host's dermis.

Because man is most frequently exposed in grassy and cleared areas, the classic vectors have been recognized to be mites inhabiting such sites. The ecological concepts, however, have been greatly expanded both in regard to environments and species of vectors: primary jungle (196), urban areas (182), mountains in Kashmir (96) and Korea (92), and Hokkaido and small islands with winter incidence in Japan (170, 190). New foci have been discovered, notably, in Japan, China, India, and the East Indies.

Though rodents are undoubted reservoirs, mites must be conceded to play an important role (143). This view is supported by the situation in the Simla Hills, India, where, after a nymphal period of about 13 weeks, mites overwinter as adults (118).

*Q fever.*—This disease is of world-wide distribution, and new foci are continuously being reported from all continents. Naturally infected tick hosts are being added so rapidly [18 species of ticks in eight genera by 1953 (200); 22 species plus 10 experimental by 1955 (185); and 52 species, including synonyms, by 1957 (191)] that lists are out of date by the time they are published. Undoubtedly, many of these records are for ticks collected directly from infected animals (115), but infected *Otobius megnini* (Dugés) have been taken from healthy cattle (90). Infected *Ornithodoros lahorensis* Neumann have failed to transmit the agent, *Coxiella burnetii* (Derrick), to guinea pigs after 19 months storage (81). All ticks do not necessarily transmit infection to their progeny (164). Only a sample of references pertinent to arthropod associations is practicable.

The reservoir mechanism for natural maintenance of *C. burnetii* is more complex than that of other rickettsial agents (145), because, in the domestic economy of man, the need for arthropod intervention in the disease cycle has been almost eliminated (entirely so in relatively "closed herd" dairy farm associations). Records of human infection following tick-bite are rare (69), yet almost any arthropods [even house flies (142)] in close contact with infected animals or contaminated premises can become carriers. The factors influencing maintenance are therefore remarkably flexible and encompass

much more than the primary natural and secondary cycles originally visualized (59, 141).

As foci diverge from congested areas to more natural environments, ticks apparently become more prominent in maintenance; e.g., Blanc (14) has stated they are the "real reservoirs" in the hills behind Casablanca, Morocco. There are many records of naturally infected, unattached ticks, including the two original foci in Queensland (33) and western Montana (55).

Contaminated dust is one source of infection. Dried tick feces can remain infectious under laboratory conditions for at least 582 days, and such stored material can cause infection by penetrating unbroken skin (142).

In southern U.S.S.R., Zhmaeva and colleagues (219) have recovered strains from three species of ticks, two of gamasoid mites, and the trombiculid, *Leeuwenhoekia major* Shluger, all of which are associated with birds. Experimental transmission of infection was accomplished by several of these, including progeny of the three ticks (216, 219). In rural areas of Czechoslovakia and in the Urals, Syrucek (188) and Burganskii *et al.* (21) consider spread in animals and thence to man to be initiated by reservoir ticks.

## Bacterial Diseases

*Tularemia.*—Because of their close association with rodents and other vertebrates that may be infected with *Pasteurella tularensis* (McCoy & Chapin), various parasitic insects and acarines are naturally infected (184). Biting flies, including mosquitoes that have caused outbreaks in the Scandinavian countries and Russia, act as mechanical vectors only. Fleas have been reported to preserve *P. tularensis* through the colder part of the year but to lose infection with the advent of warm weather (130, 199). Several species of mites have been found to be naturally infected, but experimental data of Grzhebina (84) and Olsuf'ev (129) indicate that survival of *P. tularensis* is too short to be of any significance. They found no evidence of the transstadial and transovarial passage described by Hopla (88) for the tropical rat mite. This species was unable to transmit by bite, but mice became infected by crushing mites orally. Possibly related species of mites could serve in a similar manner as sources of infection in nature.

Ticks of various species appear to be the chief reservoir in parts of the Northern Hemisphere, and isolations of *P. tularensis* from them have often been reported in the United States (31, 184, 208), including Alaska (148), France (79), Norway (101), Czechoslovakia (13), and the U.S.S.R. (131, 165, 183).

Transstadial passage by, and overwinter survival in, ticks are well established facts. Bell (9) believed organisms were purged and reacquired at each feeding. Passage of *P. tularensis* to progeny of *Dermacentor* spp. has been reported by several workers (128, 135, 150, 165), and further evidence was furnished by Calhoun & Alford (32), who isolated *P. tularensis* from unfed larval *Amblyomma americanum* (Linnaeus) in Arkansas. On the other hand, Bell (9) could not confirm transovarial transmission in *D. variabilis*, and

others have failed to mention it in demonstrating transstadial passage in other species of ticks.

*Plague.*—Observations made in the western United States and in the Soviet Union, as reviewed by Pollitzer (153) and Ioff (89), respectively, leave no doubt that rodent fleas play an important role in the maintenance of plague in nature. The reservoir potential of fleas depends primarily on the length of the extrinsic incubation period, i.e., the time elapsed from the infectious feeding to the establishment of the infective state resulting from partial to total blockage of the proventriculus. Once the blocked state has occurred, the fleas die within one to four days; according to Kartman & Prince (97), the extrinsic incubation period in *Xenopsylla cheopis* (Rothschild) has a range of 21 to 60 days at 8°C., 11 to 49 days at 21°C., and 3 to 25 days at 30°C. Wild rodent fleas, on the other hand, show a more prolonged incubation period. Under relatively cool conditions in rodent burrows, especially during the winter, some can undoubtedly carry infection into the next season.

*Brucellosis.*—In the U.S.S.R., among certain livestock, this disease is reported to have a reservoir in both ixodid and argasid ticks (156, 162). Gudoshnik (85) reports 17 species of ticks experimentally and 12 naturally infected, but he does not name them. Unfed infected ticks have been taken from pastures as well as from stock. Isolation of *Brucella* from tick species taken directly from infected animals probably reduces the significance of these findings, but *Brucella abortus* (Schmidt & Weis) was recovered from *H. concinna* off healthy cattle, and fasting *O. lahorensis* were found infected on premises where infected sheep had been kept more than a year previously (162). Studies on *Dermacentor marginatus* (Sulzer) are most informative because both transstadial and transovarial passage of *Brucella melitensis* (Hughes) have been demonstrated in naturally and experimentally infected ticks. Maximum infection persists in adults stored at room temperature up to 105 to 120 days but is reported to be reduced after 200 to 300 days. Though available data do not indicate a high percentage of transmission by *D. marginatus*, the fortuities of bacteremia in the animal host probably affect this figure. In Mexico, *Boophilus* ticks from cattle were also found to be naturally infected. Experimentally, these ticks, plus *Amblyomma cajennense* (Fabricius), bedbugs, and fleas were infected on guinea pigs, and all but the last continued to shed *B. abortus* in feces for longer than 3 months (194). Except perhaps under special local conditions, mammals rather than ticks are considered to be the important reservoirs of *Brucella*.

*Spirochetoses.*—Because reviews on louse- and tick-borne relapsing fevers have been published recently (50, 121, 202), only very selective references on the significance of ticks as reservoirs of spirochetes in nature are discussed. It is generally accepted that the human body louse, *Pediculus humanus* Linnaeus, is of no significance as a reservoir of spirochetes because of the insect's short life span, lack of transovarial transmission, and its restriction to man. On the other hand, several species of argasid ticks have been in-

criminated as basic reservoirs of spirochetes in nature because of longevity, ability to survive starvation for long periods of time, and, in many instances, transovarial passage of organisms. *Ornithodoros tholozani* (Laboulbène & Mégnin), for instance, has been shown to survive in the laboratory for as long as 20 years and to reproduce after 17, 18, and 20 years (139). The ticks preserved their spirochetes in fully virulent form and transmitted them for as long as 17 years. A specimen of *Ornithodoros erraticus* (Lucas) lived unfed for 14 years (53), and transmission of *Borrelia turicatae* (Brumpt) by *Ornithodoros turicata* (Dugés) and of *Borrelia hispanica* (de Buen) by *O. nicollei* after at least five years of starvation have been reported by Francis (73) and Davis & Mavros (57).

Several species of argasids have been found to be natural vectors (50) since the role of *Ornithodoros moubata* (Murray) as vector of the East African relapsing fever spirochete, *Borrelia duttonii* (Novy & Knapp), was established and passage of this spirochete to the ticks' progeny was proved. The remarkable specificity of species of *Borrelia* for their tick hosts has been studied most intensively in case of *O. moubata*. Burgdorfer (22) presented a detailed study on the development and behavior of *B. duttonii* and its transmission by *O. moubata;* he and other investigators (22, 121, 122, 202) discussed the controversial question of whether multiplication of spirochetes in ticks or lice was more complicated than simple transverse division.

A very detailed account on the mechanism of transovarial transmission in *O. moubata* was presented by Aeschlimann (2). He found that as many as 90 per cent of the progeny were infected, 60 per cent of which transmitted the agent to their offspring. He also observed loss of virulence of spirochetes after four generations of transovarial passage. Comparable observations were reported by Geigy & Mooser (77) and Geigy *et al.* (78) with *B. duttonii* in ticks collected in Itete (Tanganyika). These authors believed that, because of acquired immunity in the local human population, propagation of spirochetes in ticks occurred over a period of three to four years by transovarial passage only. They speculated that infection in man, the only susceptible host of *B. duttonii*, is necessary to maintain virulence, but in his absence spirochetes will eventually become avirulent in the tick population. Burgdorfer (26) found no loss of virulence in several strains of *B. duttonii* (including one believed to be a substrain of the strains studied by Geigy and associates) that were passed transovarially through five generations of *O. moubata*. This question needs further clarification, as it is pertinent to an understanding of the reservoir mechanism.

For the majority of other tick-borne relapsing fevers, rodents are the important host animals for both ticks and spirochetes. Transovarial transmission of the organisms has been shown to occur in several species of *Ornithodoros*. Infection rates of the progeny vary greatly and may be high, as in *O. turicata* (47) and *O. tholozani* (53), or low, as in *O. hermsi* Wheeler [Hermes & Meyer (87)] and *O. tartakovskyi* Olenev (5); transovarial transmission does not occur in *O. parkeri* Cooley and *O. talaje* (Guérin-Meneville) (52).

Also of importance in natural survival is the vector-spirochete "specific-

ity," whereby each species of tick preserves and transmits only its own spirochete (46). In North America, for instance, a complete restrictive specificity has been claimed between *Borrelia turicatae*, *Borrelia hermsii* (Davis), and *Borrelia parkeri* (Davis) and their respective tick vectors; this specificity has been used for classification and identification of spirochete isolates from mammals and their probable tick vectors (48, 49, 51). Exceptions to this specificity hypothesis are few but have been reported (54). In Africa and the Near and Middle East, this specificity exists even *intra speciem* (6, 56): *O. tholozani* from one locality in Iran, for instance, does not transmit *Borrelia persica* (Dschunkowsky) strains from another part of that country.

Fowl spirochetosis, a world-wide and important disease of poultry, is caused by *Borrelia anserina* (Sakharoff) and transmitted by *Argas* spp., in which the spirochetes multiply and pass through the eggs (126). As in mammalian spirochetoses, the early claim of an elaborate cyclic development in ticks has now been discredited. A dermanyssid mite has also been claimed to be a vector in Austria (126).

The livestock spirochete in South Africa, the Caucasus, and, possibly, Brazil, *Borrelia theileri* (Laveran), surprisingly, is transmitted by ixodid ticks, one-host *Boopilus* spp. and two-host *Rhipicephalus evertsi* Neumann, in both of which the organisms are transmitted transovarially and transstadially (126).

The reservoir mechanism in all of these animal spirochetoses is comprised of a combination of persisting infection in the vertebrate hosts and natural maintenance through generations of the vectors.

*Leptospirosis.*—There is little evidence that blood-sucking arthropods play a role in maintaining leptospirae in nature. Several isolations of these organisms, especially from ixodid ticks, have been reported (103, 197), but it appears that these are merely indications of leptospiremia in the blood of the animals from which the ticks were taken. Burgdorfer (25) found that *D. andersoni*, *A. maculatum*, and possibly other species of ixodid ticks could become very efficient vectors of *Leptospira pomona* (Derrick) if they had access to rather large concentrations of organisms, such as by artificial feeding. When fed on infectious laboratory animals, however, these ticks retain leptospirae only temporarily. In nature, as far as is known, leptospiremia in animals is rather mild; thus, it is doubtful that ticks feeding during this period would become permanent carriers of leptospirae. Of various ectoparasites tested experimentally (4), ticks of the genus *Ornithodoros*, particularly *O. moubata* and *O. turicata*, have been found to be most susceptible to leptospirae (23, 28, 171). However, natural infection was reported only once, by Burgdorfer (23), who isolated a strain of *Leptospira ballum* (Borg-Peterson) from a single specimen of *O. turicata*.

## Tick-Borne Protozoan Diseases

*Sporozoa.*—This group of microbial agents is one of the most important and widespread of the agents that affect the health of animals; fortunately, none of these parasites are known to infect man. They are often grouped to-

gether as Piroplasmata or Haemosporidia. A few of the better known Sporozoa are mentioned in the following paragraphs.

*Cattle.*—The only means of natural transmission of the four species of *Babesia* that cause red-water ("Texas fever") in cattle are by ixodid ticks of five genera, of which different species of *Boophilus* are the most important, depending on locale (61, 126). Transmission is complicated by the one-, two-, or three-host habits of the different species. Because cattle show premunition, they are customarily considered the primary reservoirs. Transovarial passage of the organisms, however, places ticks in an important supplemental reservoir role, regardless of the number of animal hosts they require per generation; this is especially true in colder climates, where the ticks overwinter either in the adult stage or often on the host in one-host species.

A "sexual cycle" of organisms has been described in *Boophilus* (58), and it also enhances the essential nature of ticks in the reservoir mechanism.

Anaplasmosis, another world-wide disease of cattle, is caused by two species of *Anaplasma* and transmitted by many of the vectors of *Babesia*, plus some others, as reviewed by Neitz (126). The reservoir mechanism includes premunized cattle, plus ticks as essential vectors in which there is continuity through the eggs; these acarines thus occupy a supplemental role analogous to that in *Babesia* infections. Though Lotze (112) and others showed occasional mechanical transmission by biting flies, even from symptomless carriers, no reservoir involvement of these insects is evident.

East Coast fever, a virulent disease of cattle caused by *Theileria parva* (Theiler), is transmitted by species of *Rhipicephalus* and *Hyalomma* of chiefly three-host habits (126). Recovered animals do not show persisting infection as in *Babesia* [although reinfection in mild cases has been reported (205)]. Consequently, tick vectors are important reservoirs, though *Rhipicephalus* spp. do not pass the organisms to their progeny and transstadial passage occurs only by reinfection of each stage on an ill animal. An interesting sequel is the recommendation that ticks on Uganda cattle be reduced but not entirely eradicated to maintain immunity in stock (41). *R. appendiculatus*, a three-host tick, is the principal vector-reservoir in Africa, and nymphs have transmitted infection at least 171 days after infection as larvae (205). Lewis, Piercy & Wiley (110) reported that stored adults of *Rhipicephalus neavei* Warburton infected as nymphs remained infected for 262 to 269 days. In the U.S.S.R., five species of *Hyalomma* are reported to transmit *Theileria* transstadially, but, surprisingly, only one is reported to transmit through the eggs (116). In Guadaloupe, latent infection in local cattle was not discovered until freshly imported cattle died from the disease apparently acquired from indigenous *Amblyomma variegatum* (Fabricius) (117).

Three other species of *Theileria* (or *Gonderia*) are reported to be transmitted by species of *Hyalomma* and *Rhipicephalus* (126), but two of these agents differ from *T. parva* in that they produce chronic infection and hence could be more like *Babesia* in having animals as chief reservoirs. In India, an outbreak of presumed theileriosis was caused by adults of a species of *Hya-*

*lomma*, in which transovarial infection was demonstrated, but progeny did not transmit until they had reached the adult stage (157). Transovarial transmission was reported in Indian *Hyalomma* sp. (*"savignyi"*) through five to six generations, but only adults were vectors in studies using calves reared on infection-free premises (158). In contrast, while transmission by nymphs infected as larvae occurred in Egyptian *Hyalomma excavatum* Koch, hibernating adult ticks were infectious but not their larval progeny (44).

*Horses.*—Tick-borne biliary fever is caused by *Babesia caballi* Nuttall & Strickland and *Babesia equi* (Laveran) in various parts of the world, except Australia and northern North America. Both agents are transmitted in the Old World mostly by two- and three-host species of *Rhipicephalus*, *Hyalomma*, and *Dermacentor* (126). Transovarial transmission has apparently not been demonstrated with *B. equi* in Africa, where the important two-host vector, *R. evertsi*, acquires *B. equi* as larvae or nymphs from the first horse and infects the second as adults. In the U.S.S.R., the one-host vector, *Hyalomma scupense* Schulze, infests hosts as larvae in November, but mass infection of animals does not appear until spring when the adult stage is reached (140). Circumstantially, therefore, it appears that the infection is acquired through the eggs, and further evidence was found in *Dermacentor marginatus*, which did not lose its infection when fed on immune animals (19).

In *B. caballi* infection, passage through eggs has been shown for several vector species, but transmission is chiefly by the adults, at least in South Africa. Persistence of the organisms was shown through seven generations of *Hyalomma marginatum* Koch fed on insusceptible rabbits; all of these ticks were the descendents of one female from an infected foal (1). Persistence through three to four generations of *Dermacentor* and *Rhipicephalus sanguineus*, with no reinfecting feedings, has also been shown. Since there is premunition in horses, the degree to which ticks contribute to the reservoir mechanism will depend on species and circumstances in a given environment.

*Dogs.*—Canine biliary fever is caused by two species of *Babesia*, *B. canis* (Piana & Galli-Valerio) and *B. gibsoni* Patton, that have a simple life-cycle in the vector ticks, chiefly *R. sanguineus* (173), *Haemaphysalis leachi*, and *Dermacentor* spp. (126). The disease is world-wide except for Australia and the Nearctic zone. *Dermacentor pictus* (Hermann) is reported to be an important vector to dogs during the winter in France; experimentally this and *H. marginatum* transmitted, but, strangely, *I. ricinus* and two other species did not (70). The infection is heritable in ticks, but again the enzootic emphasis is on adult tick transmission, since results of larval and nymphal tests have been irregular. Premunized dogs are not reported to be infectious for ticks, but both dogs and ticks conceivably act as reservoirs; possibly ticks are more important in colder climates.

*Trypanosomiasis.*—In Africa, although *Trypanosoma* species transmitted by tsetse flies (*Glossina* spp.) undergo a "definite cycle and multiplication" in those vectors, "mammalian reservoirs" are generally looked upon as the sources of infection (30). Occasional flies survive in cage and liberation-

marker studies for three to five months, or six at the most, under favorable conditions (190 days in one case); but population observations indicate that flies do not live this long in the field (30).

Tsetse flies would therefore appear to function as the principal reservoirs only in a supplemental role to mammals, including man. Wilcocks (204) reviewed recent evidence supporting this viewpoint, particularly in relation to *Trypanosoma rhodesiense* Stephens & Fanthan, though such evidence, largely circumstantial, varies for different species of trypanosomes (45).

Since tsetse flies are limited to Africa, but trypanosomiasis is a "major livestock scourge in every continent except Australia" (61) (mostly transmitted mechanically by biting flies, as surra by tabanids in the Far East), parasites persist in animals in wide areas without the known intervention of an intermediate biological host. One is led to wonder how correct the following statement (61) really is regarding what has been considered an accepted appraisal of the African epizootiology:

> Extensive studies of nagana seem to warrant the general deduction that mechanical vectors have a large part in the spread of African trypanosomiasis but that cyclical development in tsetse flies is essential to the perpetuation of the diseases.

In Chagas' disease, however, the role of the insect vectors, triatomid bugs, becomes more prominent in the reservoir function because of longer lifespans and persistence of *Trypanosoma cruzi* Chagas through all the active stages of the bugs in which cyclic-propagation likewise occurs. Though organisms do not pass through the eggs, the unique but common habit of harmless cannibalism on freshly fed infected bugs, especially by younger unfed bugs, can spread infection in a colony. Fecal contamination from infected bugs is probably the main means of transmission to animals in the Neotropics; this is in contrast to infection by bites of flies such as *Glossina* and *Phlebotomus* in other trypanosomiases.

In a 10-year population study in a Brazilian focus, Dias (60) found a staggered seasonal occurrence of different stages of the two chief vectors, *Triatoma infestans* (Klug) and *Panstrongulus megistis* (Burmeister), so that they straddled the season of highest disease incidence during hot, wet weather. Of five species of bugs that habitally infest human dwellings and are considered epidemiologically important vectors to man, *T. infestans* repeatedly had the highest natural rate of infection [27.69 per cent in one study by Bustamante, a rate much higher than found in African *Glossina* (30)].

These triatomids are viewed by some investigators as the important reservoirs in Chagas' disease in spite of chronicity in vertebrates (76, 207). Nine species of bugs have been listed as natural vectors (76).

In diseases in which reservoir functions are shared by both vectors and mammalian hosts, quantitative factors may influence opinions of relative importance. Neghme & Román (124) reported that 12 per cent of a random-sampled human population in endemic zones of Chile had evidence of *T. cruzi* infection, 13.4 per cent of cats and dogs, 1.6 per cent of wild mammals,

and 44.4 per cent of *T. infestans*. Such figures could be misleading, however, because population samples would naturally include more bugs than animals in a given focus.

In contrast, *T. cruzi* persists in the southern United States in indigenous bugs and rodents (187, 206) almost in absence of human infection.

## Conclusions

While vertebrate hosts are generally conceded to play the major role as reservoirs of arthropod-borne animal disease agents, it is evident that arthropod vectors often have at least a supplemental, sometimes a major, function. Facts that enhance the importance of such arthropods as reservoirs are: longevity, including overwintering or inter-epizootic survival in appropriate environments, attack on more than one vertebrate host in a given generation, adaptations that result in transmission by several stages and passage of agents through the eggs to progeny, and cyclic propagation in the vectors.

The literature reveals that the reservoir mechanism of pathogens among the viruses, rickettsiae, bacteria, and protozoa comprises not a static but a dynamic and flexible adjustment in the relative importance of arthropod and vertebrate hosts in natural maintenance of a given microbial parasite. The remarkable, often spectacular, ways in which this adaptation has evolved in the general biocoenoses of different diseases is often unappreciated. The moral of this story is: beware of comprehensive tables that formally separate "vectors" and "reservoirs" (meaning vertebrate hosts) into two columns; such tables will only provide the true biological facts for part of the examples. It also appears evident that "insects" have been living together with microbes much longer than have vertebrates (145), but that is another story.

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# TRANSMISSION OF FILARIOID NEMATODES[1]

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The chief characteristics of the filarial worms (using this term in a loose sense) are: the female worm produces embryonated eggs or larvae (microfilariae) in the body of the vertebrate host; these larvae are ingested by an arthropod in which they develop through, usually, three larval stages and increase in size; and the larvae then enter another vertebrate host, usually while the arthropod is biting. Thus, the worm utilises the arthropod both for transmission from one vertebrate to another and for larval development.

It had previously been considered [e.g., Chandler *et al.* (21)] that this life cycle had evolved through forms analogous to *Habronema*, but Anderson (2) points out that, more probably, they developed in the following manner. Originally there was a common ancestor for both the *Filarioidea* and *Spiruroidea*, which inhabited the gut of the host and had a simple spiruroid life-cycle [e.g., *Rhabdochona ovifilamenta* Weller (Thelaziinae)]. Further development was probably as follows: (*a*) The ancestors established themselves in the orbit but retained a spiruroid life-cycle, e.g., *Oxyspirura mansoni* (Cobbold) (Thelaziinae). (*b*) The larvae accumulated in lacrymal secretions, from which they were taken up by arthropods, which became vectors and carried them to the eyes of other hosts, e.g., *Thelazia*. (*c*) The adults established themselves in subcutaneous tissue and returned to the orbit to deposit their larvae (hypothetical forms). (*d*) The adults pierced the skin and released larvae which developed in haematophagous arthropods attracted to the bleeding lesion; the infective larvae left the arthropod while the latter was feeding and grew to the adult stage under the skin of a new vertebrate host, e.g., *Parafilaria multipapillosa* (Condamine & Drouilly) (Filariinae). (*e*) The larvae accumulated in ulcerated skin lesions around the female worm and were taken up by haematophagous arthropods, which carried them to other hosts where they developed in the subcutaneous tissues, e.g., *Stephanofilaria* (*Stephanofilariidae*). [Note, however, that, according to Rahman (112), the larvae of *Stephanofilaria assamensis* Pande are picked up by flies that feed on the sores and are carried mechanically on the outside of the flies to sores on other cattle, where they develop and produce new hump sores; this view requires confirmation before acceptance.] (*f*) The larvae accumulated in the subepidermis and were removed by haematophagous arthropods that could pierce the skin; thus, skin lesions associated with the early larvae became unnecessary, e.g., *Onchocerca* (*Onchocercinae*). (*g*) The larvae accumulated in the blood, and the adults became free to colonise the deeper tissues of the host, e.g., *Wuchereria* (*Dipetalonematidae*).

[1] The survey of the literature pertaining to this review was concluded in May, 1960.

## TABLE I

### A List of the Filarial Worms Known to Develop in Arthropods*‡

| Filarial worm | Principal definitive host | Arthropod host | Site of development in arthropod | Reference |
|---|---|---|---|---|
| *Aproctoides lissum* Chandler | *Francolinus pondicerianus* | *Culex fatigans* Wiedemann | Thoracic muscles | 110 |
| *Brugia malayi* (Brug)† | Man, domestic cat, certain primates | *Mansonia* spp. | Thoracic muscles | 10, 36, 135, 136 |
| *Brugia pahangi* (Buckley & Edeson)† | Domestic cat | *Mansonia uniformis* (Theobald) | | 34 |
| *Brugia patei* (Buckley *et al.*)† | Cat, dog | *Aedes pembaenis* (Theobald) | Thoracic muscles | 47 |
| *Conispiculum guindiensis* Pandit *et al.* | *Calotes versicolor* | *C. fatigans* | Thoracic muscles | 103 |
| *Dipetalonema arbuta* Highby | *Erethizon dorsatum* | *Aedes* spp., *Mansonia perturbans* (Walker) | Fat body | 50, 51 |
| *Dipetalonema grassi* (Noè) | Dog | *Rhipicephalus sanguineus* (Latreille) | Muscles | 99 |
| *Dipetalonema perstans* (Manson) | Man | *Culicoides milnei* Austen (=*austeni*), *Culicoides* spp. | Thoracic muscles | 30, 52, 119 |
| *Dipetalonema reconditum* (Grassi) | Dog | Various fleas | Fat body | 42 |
| *Dipetalonema* sp. (?*reconditum*) | Dog | *Ctenocephalides* spp. | ?Haemocoele | 93 |
| *Dipetalonema streptocerca* (Macfie & Corson) | Man | *Culicoides grahamii* Austen | Thoracic muscles | 22 |
| *Dipetalonema vitae* (Krepkogorskaya) (=*blanci*) | *Meriones libycus*, *Rhombomys opimus* | *Ornithodorus tartakovskyi* Olenev, *Rhipicephalus* sp. | Muscles | 19 |
| *Dirofilaria corynodes* (Linstow) | *Cercopithecus aethiops*, *Colobus badius* | *Aedes aegypti* (Linnaeus), *Anopheles atroparvus* van Theil | Fat body | 133 |
| *Dirofilaria magnilarvatum* Price | *Macacus irus* | *Mansonia longipalpis* van der Wulp, *M. uniformis* | Malpighian tubules | 137 |
| *Dirofilaria immitis* (Leidy) | Dog (and several other spp. of carnivores) | Several spp. of anophelines and culicines | Malpighian tubules | 43, 65, 97, 105 |
| *Dirofilaria repens* (Railliet & Henry) | Dog | *A. aegypti*, *Anopheles maculipennis* Meigen | Malpighian tubules | 5, 38 |
| *Dirofilaria scapiceps* (Leidy) | *Lepus americanus phaenotus* | *Aedes* spp. | Fat body | 50, 51 |
| *Dirofilaria subdermata* (Mönnig) | *Erethizon dorsatum* | *Aedes stimulans* (Walker) | | 49 |
| *Dirofilaria tenuis* Chandler | Raccoon | *Aedes taeniorhynchus* (Wiedemann), *Anopheles quadrimaculatus* Say | Malpighian tubules | 106 |
| *Foleyella brachyoptera* Wehr & Causey | *Rana sphenocephala*, *Rana pipiens* | *A. aegypti*, *Culex pipiens* Linnaeus, *C. fatigans* | Fat body | 16, 17, 18, 74 |

| Filarial worm | Principal definitive host | Arthropod host | Site of development in arthropod | Reference |
|---|---|---|---|---|
| *Foleyella dolichoptera* Wehr & Causey | *Rana sphenocephala, Rana pipiens* | *A. aegypti, C. fatigans* | Fat body | 16, 17, 18, 74 |
| *Foleyella duboisi* (Gedoelst) | *Rana esculenta ridibunda* | *Culex molestus* Forskål | Haemocoele | 143 |
| *Foleyella ranae* Walton | *Rana clamitans* | *A. aegypti, C. pipiens, C. fatigans* | Fat body | 16, 17, 18, 74 |
| *Icosiella neglecta* (Diesing) | *Rana esculenta* | *Forcipomyia velox* Winnertz, *Sycorax silacea* Haliday | Thoracic muscles | 26, 27 |
| *Litomosoides carinii* (Travassos) | *Sigmodon hispidus litoralis* | *Ornithonyssus bacoti* (Hirst) | Parenchyma | 6, 57, 141 |
| *Loa loa* (Cobbold) | Man, monkey | *Chrysops silacea* Austen, *Chrysops* spp. | Fat body | 23, 72 |
| *Mansonella ozzardi* (Manson) | Man | *Culicoides furens* (Poey) | Thoracic muscles | 11 |
| *Microfilaria fijiensis* Yeh *et al.* | *Pteropus hawaiiensis* | *Aedes polynesiensis* Marks, *Aedes pseudoscutellaris* (Theobald) | Thoracic muscles | 125 |
| *Onchocerca cervicalis* Railliet & Henry | Horse, mule, donkey | *Culicoides nubeculosus* (Meigen) | Thoracic muscles | 122 |
| *Onchocerca gibsoni* Cleland & Johnston | Cattle | *Culicoides pungens, Culicoides* spp. | | 12 |
| *Onchocerca gutturosa* Neumann | Cattle | *Simulium ornatum* Meigen | Thoracic muscles | 123 |
| *Onchocerca volvulus* (Leuckart) | Man | *Simulium damnosum* Theobald, *Simulium* spp. | Thoracic muscles | 8, 9, 25, 81 |
| *Ornithofilaria fallisensis* Anderson | Domestic ducks, wild black ducks | *Simulium rugglesi, Eusimulium* spp. | Haemocoele | 1 |
| *Oswaldofilaria chlamydosauri* (Breinl) | *Amphibolurus barbatus, Chlamydosaurus kingi* | *C. fatigans, Culex annulirostris* Skuse | Fat body | 82 |
| *Setaria cervi* (Rudolphi) (=*labiatopapillosa*) | Cattle, buffalo, goat, other ruminants | *Stomoxys calcitrans* (Linnaeus) | Thoracic muscles | 98 |
| *Setaria digitata* (von Linstow) | Sheep, goat, horse, zebu | *Anopheles sinensis* Wiedemann, *Aedes togoi* (Theobald), *Armigeres obturbans* (Walker) | Thoracic muscles | 120 |
| *Setaria equina* (Abildgaard) | Horse | *Tabanus tropicus* Panzer | | 80 |
| *Setaria marshalli* Boulenger | Sheep, cattle | *C. pipiens, Aedes* spp. | | 64 |
| *Wuchereria bancrofti* (Cobbold)† | Man | *Tabanus* spp. | Thoracic muscles | 3, 73, 85, 100 |

* Modified from Lavoipierre (76).

‡ After this review was sent to press, a paper by Gnedina & Osipov was seen in which the intermediate host of *Parafilaria multipapillosa* (Condamine & Drouilly) was shown experimentally to be a blood-sucking muscid fly, *Haematobia atripalpis* Bezzi. Development was in the fat body of the insect and took 10 to 15 days at 20°–36.5°C. [Gnedina, M., and Osipov, A., *Helminthologia* (In Russian), **2,** 13–16 (1960)].

† Buckley (14), after examination of numerous specimens of *Wuchereria malayi*, has erected the genus *Brugia* to accommodate this and closely related species from the cat and dog.

The classification of the *Spiruroidea* and *Filarioidea* is undergoing revision at the present time, and it is possible that the latest version [Chabaud & Anderson (20)] will undergo further modification. Accordingly, for the purpose of this review, "filarial worms" are taken as those nematodes that are transmitted by haematophagous arthropods and that undergo development from microfilaria to infective larva (i.e., 2 moults) during this transmission. It may be noted that this mode of transmission involves a long delay (up to several months) in the microfilaria stage, a period of rapid development lasting two to three weeks in the arthropod, a further period of development after entry into the vertebrate host (up to eleven months in some species), and, finally, a prolonged period of many (in some cases more than seven) years during which the adult female produces millions of microfilariae.

Excellent reviews of filarial transmission have been written by Lavoipierre (76) and, of the more limited aspects, by Kartman (70); we are much indebted to both of these researchers. Aspects that have been described in detail in these reviews are handled more briefly in our own article. Papers that describe the distribution of human infections in specific geographical areas or of the corresponding vectors have not been reviewed. A list of filarial worms, whose transmission through arthropods is known, is shown in Table I, which is reproduced, with minor modifications, from Lavoipierre (76). Most of the work on the transmission of filariae has been concerned with species of medical and veterinary importance.

Recent research has benefited greatly by the maintenance of several filarial infections in the laboratory. The two chief infections thus maintained have been *Litomosoides carinii* (Travassos) [Bertram *et al.* (7); Hawking & Sewell (45); Scott & Cross (117); Williams & Brown (142); and many others] and *Dipetalonema vite* (Krepkogorskaya) [Baltazard *et al.* (4); Wagner (131); personal observations]. *Dirofilaria immitis* (Leidy) and *Dirofilaria repens* Railliet & Henry have also been maintained [Webber & Hawking (134)], but these are more cumbersome. In addition, other infections have been passaged in the laboratory but have proved inconvenient for regular transmission. Many different kinds of mosquitoes have been found to be able to transmit the human filaria, *Wuchereria bancrofti* (Cobbold), and the ability to transmit is not in strict accordance with the systematic classification of these insects. Recent lists of vectors of *Wuchereria* have been given by Manson-Bahr (86) and Raghavan (109). A bibliography on vectors of *Wuchereria* in the South Pacific region has been published by Iyengar (60) and one on onchocerciasis (up to 1945) by the Pan American Sanitary Bureau (102); these papers should be referred to for further information. The mosquitoes known to be capable of transmitting *D. immitis* have been discussed by Kartman (70); however, his indication that this species had been transmitted by fleas is probably incorrect, because it had not then been realised that many of the microfilariae found in dogs in America did not belong to *D. immitis* but to a species of *Dipetalonema* that resembles *D. reconditum*

(Grassi); the adults, however, show slight differences, and it is not yet decided whether the two are identical [Newton & Wright (93, 94)]. This demonstration of two kinds of filarial worm occurring in dogs in North America may remove some of the confusion that has arisen there about the vectors of "*D. immitis*." The interesting observations of Stueben (124) about filarial worms in fleas presumably refer to *Dipetalonema reconditum*, and not to *Dirofilaria immitis* as he supposed.

The biological purpose of transmission is to convey the filarial worm from one host to another and to allow certain stages of development to occur during this process. Accordingly, the subject can be considered under the headings:

(*a*) Passage of microfilaria from vertebrate into arthropod host.
(b) Development within arthropod host
  (i) Passage from lumen of gut to specific site in the tissues.
  (ii) Development in specific site.
  (iii) Passage from specific site to proboscis.
(*c*) Passage of infective larva from arthropod to vertebrate host.

## PASSAGE OF MICROFILARIA INTO ARTHROPOD VECTOR

The first stage of the transmission of a filarial worm is obviously the passage from the vertebrate host into a blood-sucking arthropod [see also Lavoipierre (76)]. This passage is influenced by many factors, such as the distribution of the microfilariae in the subcutaneous tissues or capillaries of the vertebrate host, the periodicity of the microfilariae, etc. Thus, *Culicoides grahami* Austen becomes infected with the microfilariae of *Dipetalonema streptocerca* (Macfie & Corson) if the midges are fed on the back and chest of a patient where worms are present, but they do not become infected if fed on areas where worms are difficult to demonstrate [Duke (29)]. In the case of periodic microfilariae, the number taken up by the mosquitoes varies, as would be expected, according to the point of the cycle at which feeding occurs. *Chrysops silacea* Austen and *Chrysops dimidiata* van der Wulp are diurnal in their feeding habits, and they tend to be infected with the human *Loa loa* (Cobbold), which is diurnal in its periodicity; in contrast, *Chrysops langi* Bequaert and *Chrysops centurionis* Austen bite at dusk or early night, and they tend to be infected with the *Loa* of monkeys or drills, which have a nocturnal periodicity [Duke (31, 32)]. Similarly, there is a close relationship between the timing of the microfilarial peak in the blood and of the maximum biting activity of the vector in *Ornithofilaria fallisensis* Anderson and its *Simulium* hosts, both of which occur about the middle of the day [Anderson (1)]. The chances of a worm, e.g., *Loa*, passing from vertebrate host to arthropod vector, e.g., *Chrysops*, are also influenced by the height above the ground in the forest canopy at which both occur [Crewe & O'Rourke (24)]. There is a suggestion that certain microfilariae tend to pass into certain blood suckers more readily than do others; thus, Chardome & Peel

(22) and Henrard & Peel (48) reported that, if *Culicoides grahami* feed on a man containing both *Dipetalonema streptocerca* and *Dipetalonema perstans* (Manson), only microfilariae of *D. streptocerca* are picked up. Conversely, *Simulium albivirgulatum* Wanson & Henrard ingested microfilariae of *D. perstans* and *Onchocerca volvulus* (Leuckart) but not of *D. streptocerca*. [There have been several papers on the identity of the vector that transmits *D. perstans;* British workers Hopkins & Nicholas (52) and Duke (30) consider that it is transmitted by *Culicoides milnei* Austen (=*austeni*) and also by *C. grahami* and *Culicoides inornatipennis* Carter, Ingram & Macfie, while Belgian workers Chardome & Peel (22) and Henrard & Peel (48) had previously suggested that such apparent transmission was erroneous and was caused by the fact that *D. streptocerca* was also present in the donors used for the investigation.] Apparently after microfilariae (*D. immitis* and *Foleyella* Seurat) leave the uterus of the female worm, they undergo an ageing process in the circulating blood for a probably short period, during which time they become more infective for their mosquito hosts [Kartman (65)].

The quantitative relationships between the number of microfilariae in the blood and the number taken up by the vector have been studied by many workers [Duke (30); Duke *et al.* (33); Kartman (65); Kershaw *et al.* (71); Nicholas & Kershaw (96); Wharton (135, 136)]. Briefly, the actual uptake is extremely variable, and it is usually less than might have been expected from the volume of blood ingested; the variation, in some cases, is caused by the variation in the number of microfilariae present in individual cutaneous capillaries; in other cases, the lower number taken up may be attributed to the vector feeding from a pool of blood that is escaping from ruptured capillaries, the erythrocytes escaping more rapidly than microfilariae. On the other hand, *Culex fatigans* Wiedemann feeding on patients with *W. bancrofti* in Malaya seemed to take up three times as many microfilariae as would have been expected from their blood meal [Wharton (138)]. There is no evidence of a chemotropic factor that attracts microfilariae to the bite, as had been postulated by some earlier workers. Some vectors, e.g., *Anopheles quadrimaculatus* Say feeding on dogs containing *D. immitis*, expel a drop of fluid from the anus, which may sometimes contain microfilariae up to 17 per cent of the number ingested, but this loss *per anum* is not considered to be of quantitative importance [Kartman (66)]; in some cases, fluid may be expelled while microfilariae and erythrocytes are retained. The same phenomenon has been studied in mosquitoes by Reid (113) and in *Simulium* by Wanson (132) and Lewis (81).

Particular attention has been directed to the question of the minimum density of microfilariae in a host that will still infect a vector. This is of great practical importance during public health campaigns planned to reduce the microfilaria reservoir in patients and carriers by the administration of diethylcarbamazine. Thus, when *Aedes polynesiensis* Marks feed on patients

with the Pacific type of *W. bancrofti* and the blood contains only one microfilaria per 50 c.mm., about 5 per cent of the mosquitoes become infected, each mosquito usually containing only a single larva [Rosen (114)]. Similarly, when *Culex pipiens fatigans* Wiedemann feed on patients containing *W. bancrofti* in Africa and the blood contains one microfilaria per 40 c.mm., about 2.5 per cent of the mosquitoes become infected [Jordan (61)]. When *Mansonia longipalpis* van der Wulp mosquitoes were fed on patients containing one microfilaria of *Brugia malayi* (Brug) per 40 c.mm. blood and were dissected 11 days later, 10 per cent of the mosquitoes each contained a single infective larva [Wharton (136)]. There is probably no absolute lower limit to the concentration of worms in the blood that may still infect mosquitoes, but the problem is a statistical one—the fewer the microfilariae in the blood, the fewer the larvae that develop in mosquitoes. When the infectivity becomes so low that even under good laboratory conditions only about two larvae develop in 100 mosquitoes, it is probable that little transmission would actually take place in the field. In a few suggestive experiments, Kartman (67) allowed *A. quadrimaculatus* to feed on microfilariae of *D. immitis* suspended in sugar solutions; later the microfilariae were found in the diverticulum, but they did not develop. *Aedes aegypti* (Linnaeus) were fed with microfilariae suspended in saline, and the worms subsequently showed development (four mosquitoes only). It would satisfy curiosity if these experiments were repeated.

## DEVELOPMENT WITHIN ARTHROPOD HOST

### Passage from Gut to Specific Site

After the microfilariae reach the stomach of the arthropod, some begin to migrate toward the body cavity within a few minutes. Microfilariae of *B. malayi* have been seen actively passing through the stomach wall of *M. longipalpis* into the abdomen within 10 minutes of feeding [Wharton (135)]. They pass through the wall of the stomach, and not through the cardiac region of the mid-gut. Practically all those that pierce the stomach will migrate forward into the thorax within 12 hr.; however, other microfilariae stay in the stomach for many hours. Larvae of *D. immitis* may be found in the gut of *A. aegypti* after 96 hr. still alive and apparently normal [Kartman (65)], but probably such stragglers cannot still complete their development. The microfilariae of what was presumed to be *D. reconditum* penetrate the gut wall and enter the abdominal haemocoele of fleas as early as 1 hr. after ingestion [Stueben (124)]. The microfilariae of *Litomosoides carinii* apparently are slow to leave the gut of *Ornithonyssus bacoti* (Hirst), but many do so during the first 24 hr.; apparently some can remain in the gut for several days and then complete their development [Freer (37)]. In this mite, there are large vacuolated cells lying in the lumen of the gut that may ingest and digest the microfilariae during the first 24 hr. [Hughes (57)].

The time of survival of microfilariae in the lumen of the gut varies according to the species of mosquito or other arthropod host. Thus, the larvae of *D. immitis* survive for 24 hr. in the gut of *A. quadrimaculatus* and they rapidly migrate to the tubules (this mosquito is a good vector and no clotting of the stomach contents occurs for several hours); in *A. aegypti* (which is a moderate vector of *D. immitis*), they survive apparently unchanged for 48 hr. The reason for the longer survival of microfilariae in some mosquitoes than in others is not clear. The rate at which they can migrate and escape from the gut is probably influenced by the speed with which the blood becomes coagulated after it has been ingested by the mosquito. If *A. aegypti* is fed on infected blood containing an anticoagulant, twice as many worms reach the tubules as in mosquitoes fed directly on an infected dog [Kartman (65)].

In the case of polymorphic trypanosomes ingested by tsetse flies, the condition of the peritrophic membrane is very important in determining whether or not the parasites will be able to establish themselves in their insect host. With filariae, this membrane seems to have received little attention, apart from the work of Lewis (81) with *Simulium damnosum* Theobald. After the formation of the peritrophic membrane, only the microfilariae (*Onchocerca volvulus*) that remain in the anterior (tubular) portion of the mid-gut are able to pass backwards into the space between the membrane and the gut wall in order to pierce the stretched wall of the distended stomach. In heavy infections of *D. immitis* in *A. aegypti*, the microfilariae may damage the peritrophic membrane so much that it is impossible to demonstrate it [Lavoipierre (76)].

In connection with the penetration of the stomach wall, it is remarkable that little attention has been paid to the presence of the hooklike structure that is present at the anterior end of most microfilariae. It has been studied particularly in *L. carinii* by McFadzean & Smiles (83) and in *D. immitis*, *D. repens*, *Dirofilaria corynodes* (Linstow), *W. bancrofti*, and *B. malayi* by Taylor (127). It has also been figured in the case of *W. bancrofti* and other filariae in the older literature, but the optical techniques of those days were not sufficient to show its true nature. This "hook" is a small, apparently sharp structure which can easily be seen in microfilariae by phase-contrast microscopy. During life it is constantly being protruded and retracted. Inside the sheath it can hardly serve any useful purpose, so presumably it must come into play after unsheathing; i.e., in the stomach of the arthropod, where it might well be used to bore its way through the wall of the stomach or into the cells of the tubule. But nothing has yet been reported about it during this phase.

Most microfilariae migrate from the lumen of the gut to their sites of development by penetrating through the wall of the mid-gut, but a few [*D. immitis*, *D. repens*, and *Dirofilaria tenuis* (Chandler)] migrate into the Malpighian tubules as a route into the body cavity. There must be some mechanism which attracts such microfilariae toward the openings of the tubules, and, presumably, the worms are guided by a chemical stimulus.

Fülleborn (39) reported evidence of a chemotaxis that attracted *D. immitis* into capillary tubes containing Malpighian tubules, but this experiment has apparently never been repeated, although it well deserves repetition.

### Development in Specific Site

Having left the gut of the arthropod, the larval nematode develops in some special site, which varies according to the species concerned. Development of filariae has been described as taking place in the muscles (especially thoracic), in the fat body (usually abdominal, but in some cases the thoracic or cephalic), and in the Malpighian tubules [Lavoipierre (76)]. Filariae have also been reported in the haemocoele, but, as this is not based on histological evidence, Lavoipierre (76) doubts it and suspects that, in many of these cases, the worms really developed in the fat body. It has recently been recognised that, in many cases, the early development occurs inside the cells rather than between them; e.g., *Loa loa* (Cobbold) [Lavoipierre (75); Lebied (78)], *B. malayi* and *W. bancrofti* [Iyengar (58, 59)], *O. volvulus* [Lebied (77)], *L. carinii* [Hughes (57)], *D. reconditum* [Grassi & Calandruccio (42)], *D. immitis* [Noè (97); Taylor (128)], and *D. vite* (personal observations). Probably most species would be found to pass through an intracellular stage if studied in detail. Iyengar (59) maintains that the larvae of *Wuchereria* in mosquitoes feed by absorbing food through their cuticle and not at the expense of the thoracic muscle.

During this stage, the larva becomes slightly shorter and much wider, developing into the well-known "sausage" form. *D. vite* seems to be unusual, since it does not pass through this "sausage" stage. In the present review, no attempt will be made to consider the structural changes that take place in filariae during their development from microfilaria to infective larva; recent papers on this subject have been published by Anderson (1), Buckley (13), Gunewardene (44), and Taylor (128). It may be noted, however, that Iyengar (59) believes that, in *Wuchereria*, the G 1 cell becomes the genital primordium, the G 2 and 3 cells give rise to the intestine, and only G 4 forms the rectum; this differs from the previous interpretation that all four G cells were employed in building up the rectum. Furthermore, although the morphological appearances have been carefully described in many worms, little or nothing has yet been done to investigate the mechanisms behind these changes that determine the sequence and the co-ordination of the different developments. Probably some of the changes are stimulated by hormones produced by special cells, as indicated by Somerville (121), for the initiation of moulting in *Trichostrongylus axei* (Cobbold). In the larva of *D. immitis*, the cells around the anal pore become much larger than would seem appropriate for their later development, and they look quite like gland cells which might well produce some hormone [Taylor (127)]; but nothing definite is yet known about such possible hormones.

*Factors that determine whether development will occur.*—As is well-known, the susceptibility of different arthropods to filarial infections varies greatly.

Kartman (68) has attempted to express the efficiency of any particular host-filaria combination mathematically. He suggested the following:

$$\text{host efficiency ratio} = \frac{\text{mean number of third-stage larvae per surviving mosquito}}{\text{mean number of microfilariae per mosquito in samples shortly after feeding}}$$

Similar factors have been worked out by Wharton (135). But an insect with a high efficiency ratio in the laboratory may not necessarily be an important vector in nature, for the season at which the insect is numerous may fail to coincide with the season when microfilariae are available in the vertebrate host [Anderson (1)].

The factors that determine whether or not microfilariae will develop in a certain arthropod are mostly obscure. Some have already been considered above. For the intracellular and later development of the worm, there must presumably be some "fit" or agreement between the surface (antigenic) pattern of the worm and the cellular pattern of the vector. But this "agreement" between parasite and vector seems to be distributed capriciously. Certainly it does not follow taxonomic lines, for (as Table I shows) different species of *Dipetalonema* will develop in a mosquito (*Aedes*), a tick (*Rhipicephalus*), in *Culicoides*, and in fleas, while *W. bancrofti* will develop in some species of *Culex* or *Anopheles* but not at all in others. Even more capricious is the development of *D. immitis*, which, on the one hand, is so catholic that it will develop in various anopheline and culicine mosquitoes but, on the other hand, is so particular that it will not develop in certain strains of *A. aegypti*, although it does develop in others. This situation has been noted by Kartman (65, 68), Le Corroler (79), Roubaud (115), and Roubaud & Colas-Belcour (116), and it has been further studied in our own laboratory; it deserves general notice. On several occasions, we found that a strain of *A. aegypti*, which was initially a transmitter of *D. immitis*, had ceased to be at all susceptible. Finally, we collected strains of *A. aegypti* from nine countries and tested them for susceptibility to infection with *D. immitis*. In three strains, the worms developed to the infective stage; in the others (including a strain from Florida which Le Corroler had found to be very susceptible), no full development occurred, although the worms reached the Malpighian tubules. It appears that the susceptibility to *D. immitis* is an unstable characteristic in *A. aegypti*. In any case, according to Mattingly (87), the species *A. aegypti* is really a complex entity containing at least three varieties. Another example of strain specificity is given by Newton, Wright & Pratt (95), who found that a United States strain of *Psorophora confinnis* (Lynch Arribálzaga) had an 80 per cent infectibility rate for *W. bancrofti*, but a Puerto Rican strain had an infectibility rate of only 12 per cent. Apparently it is not enough to specify the genus and species of a mosquito as a vector of filariae, one must even specify the strain as well (at any rate, with some mosquitoes). It would be interesting to compare susceptible and non-susceptible strains of *A. aegypti* to see if the susceptibility could be linked to the presence or absence of some chemical

substance, e.g., a certain amino acid. Preliminary studies along these lines have been made with *O. bacoti* by Zein-Eldin & Scott (147, 148). Kartman (65) found that artificial selection resulted in the establishment of strains of *A. aegypti* more susceptible and less susceptible, respectively, to *D. immitis* [for further review see Kartman (70)].

The influence of the diet of the arthropod upon the development of the filariae has received little attention. If the protein requirements of the developing larvae are appreciable, compared with the size of the insect, then mosquitoes infected with filariae should survive better if a second blood meal is given. In the case of malaria parasites developing in *A. aegypti*, the feeding of acids, bases, or phosphates to the mosquitoes alters the number of oocysts that develop [Terzian & Stahler (129)]; it would be interesting to investigate whether such dietary influences would affect the development of filariae also. The development of *D. repens* in mosquitoes is not inhibited by the administration of diethylcarbamazine in their glucose [Hawking, Sewell & Thurston (46)].

The effect of temperature on development has been reviewed by Kartman (70). Briefly, there is an optimum temperature at which the maximum number of mosquitoes become infective and survive, a minimum temperature below which development does not occur, and a maximum temperature above which most arthropods die. These probably vary with different worms. With *Ornithofilaria fallisensis* in *Simulium*, development to infective larvae occurred in 14 days at 17°C. [Anderson (1)]. With *W. bancrofti* in *C. pipiens*, the microfilariae were incapable of penetrating the haemocoele at temperatures ranging from −1° to 14°C. [Hu (54)]. *W. bancrofti* in *Culex pipiens pallens* Coquillet developed to the infective larva stage in 12 days at 27°C. and in 15 days at 25°C. [Omori (101)]. In a tropical climate, the complete development of *D. immitis* in *A. aegypti* occurs in 10 to 15 days, but at a constant temperature of 27°C. it took 15 to 20 days [Le Corroler (79)]. *D. repens* in *Aedes albopictus* (Skuse) in Ceylon developed to infective stage in 9 to 10 days [Gunewardene (44)]. The rate of development also depends upon how suitable the mosquito is for the worm. Thus, at 24°–27°C., *D. repens* developed in *Anopheles stephensi* Liston (a very favourable host) in 10 to 11 days, in *Anopheles maculipennis atroparvus* Van Thiel in 12 to 13 days, and in *A. aegypti* (a borderline host) in 13 to 21 days [Webber & Hawking (134)]. The development of *D. immitis* in *A. aegypti* is not affected by changes of atmospheric pressure equivalent to sea level and 18,000 ft. [Williams (140)]. The presence of one infection in a mosquito does not prevent the development of a second infection from later blood feeds; there is no suggestion of immunity reactions by the mosquito or inhibition by the first brood of parasites [Byrd, St. Amant & Bromberg (15); Feng (35); Hu (53); Hu & Chang (56); Phillips (105)]. The presence of malaria parasites (*Plasmodium relictum* Grassi & Feletti) in *C. pipiens fatigans* does not antagonise the development of lizard filariae, *Conispiculum guindiensis* Pandit, Pandit & Iyer [Raghavan & Misra (111)].

One method of studying the conditions necessary for the development

of filariae would be to attempt to culture them *in vitro*. So far, this has been done only to a small extent. Preliminary experiments have been made with *D. immitis* by Joyeux & Sautet (63), with *O. volvulus* by Vargas (130), and with various filariae by Taylor (126). Microfilariae of *D. immitis* survived (50 per cent of them) for six days in the allantoic sac of the developing chick embryo [Mann & Fratta (84)]; this probably has no special significance, since microfilariae will survive without development *in vitro* in serum and in Ringer glucose at 37°C. for up to 14 days, but this is only survival and not development.

Workers on the development of other parasites in the arthropod vectors have studied the effect of sidetracking the passage through the alimentary canal by injecting the parasites directly into the haemocoele. Thus gametocytes of *Plasmodium gallinaceum* Brumpt have been injected into the haemocoele of *A. aegypti*, and growth of oocysts occurred in some cases, showing that intestinal phase was not essential. Similarly, *Trypanosoma rhodesiense* Stephens & Fantham injected into tsetse flies multiplies in the haemocoele as crithidia (unpublished experiments by the authors). Similar experiments with the proper vectors of filariae have not been reported, but Yoeli, Alger & Most (144) injected microfilariae of *D. immitis* into the haemolymph of wax moth larvae [*Galleria mellonella* (Linnaeus)]. At 27°–30°C. they developed for four or more days up to the "sausage" stage, and then they halted at a stage corresponding to the fifth to sixth day stage of mosquitoes. No infective larvae were ever found. Cellular reactions, e.g., encapsulation and giant cell formation, occurred around the larvae, as it does in the case of mosquitoes, when development was arrested. This partial success suggests that the importance of "fit" between filaria and arthropod is less specific than is usually supposed. In the same experiments, if the wax moth larvae were kept at 37°C., the microfilariae showed no metamorphosis or development (although they lived up to 11 days), indicating that reduction in temperature is one of the important factors stimulating the microfilaria to begin its further development.

*Effect of larva upon arthropod.*—The presence of larvae in more than a limited number usually has a harmful effect upon the arthropod host. This aspect of transmission has been well reviewed by Lavoipierre (76). Histological evidence of damage has been demonstrated in the following instances: *D. immitis* and *D. repens* in Malpighian tubules of mosquito by Noè (97), Rosen (114), and many others; *Onchocerca cervicalis* Railliet & Henry in muscle fibres of *Culicoides* [Steward (122)]; *O. volvulus* in muscles of *Simulium* [Lebied (77)]; *D. vite* in muscles of tick [Chabaud (19)]; and interference with formation of peritrophic membrane in *O. volvulus* in *Simulium* and *D. immitis* in *A. aegypti* [Lavoipierre (76); Lewis (81)]. This damage (especially that to muscle fibres) may seriously affect flight. Escape of the infective larvae may cause injury to the mouth parts, cutting short the life of the arthropod after it has transmitted its filarial parasites [Pratt & Newton (108); and others]. The infective larvae of *W. bancrofti* in *C. pipiens*

*pallens* have a "penetrating habit"; besides making their way into the proboscis, they also force their way into femora, tibiae, basal segments, maxillary palp, etc; in some cases, they thrust their cephalic end into narrow openings and then seem unable to withdraw, so that they die in this position. These penetrations of the larvae may cause abnormal behaviour of the mosquitoes, e.g., resting on the bottom of the cage, walking about, jumping suddenly and haphazardly, or inability to fly [Omori (101)]. The damage caused by the filaria is also manifested by the increased mortality of the infected vectors, which is related to the number of larvae ingested; a few larvae have no effect, more larvae cause an increasing mortality, which is proportional to the number of larvae ingested; eventually, at a certain number of larvae, all the vectors (mosquitoes) are killed. In the case of *D. repens* developing in *A. maculipennis atroparvus*, as many as 200 "small sausage" forms may be found in mosquitoes dying after six days, or 100 "late sausage" forms in mosquitoes dying after 9 days; with such heavy infections, it can easily be understood that the Malpighian tubules suffer much damage [Webber & Hawking (134)]. With *D. tenuis* developing in *A. taeniorhynchus* and *A. quadrimaculatus*, there are two peaks of mortality of the mosquitoes during the second day and during the eighth and ninth days, which Pistey (107) suggests are caused by the migration of the larvae into the Malpighian tubules and by their bursting out therefrom, respectively. Similar suggestions have been made by other workers, but Lavoipierre (76) considers that many of the conclusions drawn are based on supposition, since they have not been supported by histological observations.

*Effect of arthropod on larva.*—The best-known effect of the arthropod upon the larva is "encapsulation," during which the worm becomes coated with a brown deposit, which eventually forms a capsule around it. Although it was originally thought that this process occurred only around dead parasites, it has now been shown that it may also take place around living worms; part of the larva is encapsulated, while other parts remain motile [Highby (50); Hu (55); Kartman (65)]. Encapsulation occurs more readily with some mosquitoes, e.g., *A. aegypti*, than with others, e.g., *Culex quinquefasciatus* Say. With *D. immitis* it occurs mainly on microfilariae and rarely on "sausage-stage" larvae; the longer the microfilariae stay in the mosquito, the greater the proportion of encapsulated forms [Kartman (69)]. This subject has been reviewed by Lavoipierre (76) and Kartman (70).

### Passage from Specific Site to Proboscis

After the infective larva has completed its development, it migrates from its selected site toward the proboscis. The larva is able to migrate freely from abdomen to head and also back again. Newton & Pratt (92) introduced the larvae of *W. bancrofti* into the abdomen of mosquitoes and soon afterwards found that many of them had migrated to the thorax and head; in one case, a larva appeared in the proboscis within five minutes of its insertion into the abdomen, so that migration must be easy and rapid. Similar results have been

obtained with *B. malayi* inserted into *M. longipalpis* [Wharton (135)]. Migration of *Loa loa* in *Chrysops silacea* is very rapid, and infective forms probably travel from the abdomen to the head and mouth parts even while the fly is feeding [Gordon & Crewe (41)].

The paths of the infective larvae of *L. loa* migrating to the head of *C. silacea* have been studied anatomically by Lavoipierre (75). These larvae pass from the thorax to the head via a large ventral cervical haemocoelic canal or by squeezing between the oesophagus, the ventral nerve-cord, and the cervical tracheal trunks; in the head, they enter a large haemocoelic space (the subcibarial haemocoelic space), which lies posterior to the cibarium. The number of larvae that can be accommodated in the head and proboscis of *Chrysops* is surprisingly large; counts of 50 to 100 larvae are common, and, in one case, 200 were seen [Gordon & Crewe (41)]. On the other hand, larvae of *Loa* never penetrate into the air-sacs of *Chrysops* [Lavoipierre (75)]. Possibly there is some factor (e.g., chemical or thermal differences in the proboscis) that influences this general *Wanderlust* of the larvae, so that they go especially to the head and proboscis; but it certainly does not guarantee that all larvae will reach their proper goal. In view of this facility for the larvae to migrate to (and from) the head, it has been recommended that, to estimate infective indices during dissections, the count should not be limited to infective larvae present in the head but should include all infective larvae anywhere in the body [Jordan (62)].

## PASSAGE FROM ARTHROPOD TO VERTEBRATE

In order to pass from arthropod vector to vertebrate host, the infective larva must (*a*) emerge from the arthropod and (*b*) penetrate the skin of the vertebrate. There is apparently no record of transmission by the vertebrate host eating the arthropod. Cotton rats did not become infected when they swallowed *O. bacoti* containing *L. carinii* [Scott & Macdonald (118)], nor did *Meriones lybicus* Lichtenstein (jirds) when they swallowed larvae of *D. vite* [Baltazard *et al.* (4)]; no transmission of *D. perstans*, *L. loa*, *O. volvulus*, *D. streptocerca*, or *W. bancrofti* across the placenta to the foetus could be found in 104 African women [Peel & van Oye (104)].

*Emergence from arthropod.*—[See Lavoipierre (76).] The exact route may well differ with the various arthropods and nematodes. The case of worms escaping from mosquitoes has received the most study. The process of emergence has never been directly observed in the feeding insect actually taking a blood meal, and opinions on the exact site of escape of the worms have been based only on observations made either on preserved material or on artificially fed flies. Lavoipierre considers that the filarial larvae probably escape from the tip of the labellae rather than through Dutton's membrane, which stretches across from the base of one labellum to the other. In the case of *Chrysops* infected with *Loa*, the larvae escape only when the labium is bent, since the mothparts are then forced into the skin; probably the larvae emerge by penetrating the membrane surrounding the base of the

labium, and the escaping larvae have been seen swarming down the bared fascicle below the collapsed labella [Gordon & Crewe (41)]. The infective forms of *O. volvulus* in *Simulium* probably escape through the concertina-like membrane on the inner surface of the labella [Gibbins (40)]. *Dipetalonema perstans* in *C. milnei* escapes from the labellum [Sharp (119)]. *Icosiella neglecta* (Diesing) has been seen emerging from the root of the mouthparts of a midge, *Forcipomyia velox* Winnertz [Desportes (26, 27)]. Judging by these examples, most filariae leave their arthropod hosts through some component of the mouthparts or from their base where they are united to the head by thin membranes.

Several kinds of stimuli seem to be concerned in causing the filariae to emerge from the arthropod; e.g., warmth of the host, the taking of a blood meal, the resultant increase in intracoelomic pressure, and the bending of the labium (at any rate in *Chrysops*). In *Chrysops*, Gordon & Crewe (41) observed that larvae emerged readily even when the flies were fed on the cold dry surface of dead mammalian tissue, so that warmth and moisture are not necessary in all cases. On the other hand, Kartman (70) consistently obtained the spontaneous emergence of larvae of *D. immitis* from the proboscis of *A. aegypti* when a saline solution containing an anaesthetised intact mosquito was gently warmed. Infective larvae of *W. bancrofti* tend to leave mosquitoes even when no blood meal is taken [Wharton (136)].

*Penetration of skin by larvae.*—The larvae, having escaped from the arthropod, are deposited on the skin around the area where the arthropod has bitten. There has been controversy as to whether they can penetrate the unbroken skin or can enter only through the puncture made by the fly. It was once thought that larvae could pass through the skin, but this seems to happen rarely, if at all. Gordon & Crewe (41) found that the larvae of *L. loa* fail to penetrate unbroken human skin, but that they readily enter through the punctures made by *Chrysops* while probing or feeding; they pass down the tunnel previously cut by the mandibles and maxillae of the fly and spread themselves out in the deeply lying haemorrhage that the fly has caused. Apparently, the position is the same with *W. bancrofti* because Yokogawa (145, 146) and Menon & Ramamurti (88) placed infective larvae on human and animal skin and found no evidence of penetration, unless the surface was broken by wounds or punctures, such as those made by feeding mosquitoes. Iyengar (59) considers that penetration occurs only when the larva is in the labium of the mosquito. Infective larvae of *Foleyella brachyoptera* Wehr & Causey failed to penetrate the skin of frogs [Kotcher (74)], and Stueben (124) found that larvae of what was presumed to be *D. reconditum* from fleas did not penetrate the intact skin of a newborn rat, although he could demonstrate them in skin punctures caused by the feeding of fleas.

While discussing this question, it may be remarked that most transmissions of filarial infections in the laboratory have been made by the artificial insertion of infective larvae under the skin of a new host. The few cases in which transmission has been produced by the natural mechanism of allowing

the arthropod to feed include the transmissions of *Dirofilaria scapiceps* (Leidy) by *Aedes* [Highby (51)], *B. malayi* by *Mansonia uniformis* (Theobald) to cats [Wharton *et al.* (139)], *D. immitis* by *A. quadrimaculatus* [Newton (91)], and *L. carinii* by *O. bacoti* (routine practice in many laboratories).

## MISCELLANEOUS ITEMS

*Identification of third-stage larvae.*—Workers in the field, who are dissecting wild mosquitoes to determine the extent to which human or other filarial infections are being transmitted, are often faced with the problem of identifying any infective larvae which may be found to decide whether they are of human or animal origin. This problem has been studied in particular by Nelson (90), who gives a key for the differentiation of eleven species of filariae. Valuable guidance is also given by Wharton (135). Nelson (89) also describes a valuable technique for preserving freshly caught mosquitoes or other arthropods until they can be dissected in the laboratory. The mosquitoes are preserved in 80 per cent ethyl alcohol. When convenient, they are transferred gradually to water, stained for three days in Mayer's acid, haemalum differentiated three days in distilled water, and then transferred to glycerine to await dissection for larvae.

TABLE II

KEY TO THE DIFFERENTIATION OF INFECTIVE FILARIAL LARVAE IN MOSQUITOES*

| | |
|---|---|
| 1.—Caudal papillae or protuberances present | 2 |
| —Caudal papillae or protuberances not present | 9 |
| 2.—Length more than 1100$\mu$ | 3 |
| —Length less than 1100$\mu$ | 6 |
| 3.—Three equal bubble-like papillae | *Wuchereria bancrofti* |
| —Three caudal papillae of various shapes and size | 4 |
| 4.—Terminal or dorsal papilla prominent | 5 |
| —All three papillae poorly developed; anal ratio less than 4.5 | *Brugia malayi and B. pahangi* |
| 5.—Terminal dorsal papilla "dog's nose" shape; lateral papillae poorly developed; larva narrows between anus and extremity; anal ratio averages 4.5 | *Brugia patei* |
| —Terminal papilla large and central; two small lateral subterminal alae; anal ratio less than 3 | *Setaria equina* |
| 6.—Anus more than 50$\mu$ from extremity | 8 |
| —Anus less than 50$\mu$ from extremity | 7 |
| 7.—Three small terminal papillae | *Dirofilaria corynodes* |
| —One small terminal papilla with or without two very small subterminal papillae | *Dirofilaria immitis* or *D. repens* |
| 8.—Three prominent terminal papillae | *Dipetalonema arbuta* |
| 9.—Length usually less than 1100$\mu$; anus less than 50$\mu$ from extremity | *Foleyella spp.* |
| —Length 1000$\mu$–1250$\mu$; anus more than 50$\mu$ from extremity | *Conispiculum flavescens* |

* Modified from Nelson (90).

*Radioactive tracing of filarial larvae.*—As an item of interest, it may be mentioned that infective larvae of *W. bancrofti* and *Setaria digitata* (v. Linstow) containing radioactive $^{32}P$ have been produced by allowing the microfilariae to develop in mosquitoes that had been made radioactive by rearing the mosquito larvae in baths containing radioactive phosphate. The development of the filarial larvae was not seriously affected by the large radiation dosage. The distribution of the $^{32}P$ in the different organisms could be studied by autoradiography, and the infective filarial larvae could be traced in the tissues of the definitive host [Dissanaike *et al.* (28)].

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